INVESTIGATION OF MOLECULAR MECHANISMS REGULATING DRUG-DRUG INTERACTIONS IN ANTIRETROVIRAL THERAPY

Thesis submitted in accordance with requirements of the University of Liverpool for the degree of Doctor of Philosophy

by

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This thesis is the result of my own work. The material contained within the thesis has not been presented, either wholly or in part, for any other degree of qualification.

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I could not ask for more.

Abbreviations

^{3}H	Tritium
µCi	Microcurie
µg	Microgram
µl	Microliter
µM	Micromolar
ABC	ATP-binding cassette
AAG	$alpha_1$- acid glycoprotein
ACN	Acetonitrile
AhR	Aryl hydrocarbon Receptor
AIDS	Acquired Immunodeficiency Syndrome
API	Active pharmaceutical ingredient
ART	Antiretroviral therapy
ARV	Antiretroviral drugs
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ATT	Anti-tubercular treatment
ATV	Atazanavir
AUC	Area under the curve
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
CAR	Constitutive androstane receptor
^{o}C	Degree Celsius
cm	Centimetres
C_{max}	Maximum plasma concentration
C_{min}	Minimum plasma concentration
CNT	Concentrative transporters
COMT	Catechol O-methyl transferase
CO_2	Carbon dioxide
CYP	Cytochrome P450
DDI	Drug-drug interactions
DMEM	Dulbecco's modified Eagle's medium

DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DRV	Darunavir
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
ENT	Equilibrative transporters
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
FDC	Fixed Dose Combinations
FUMC	Fumitremorgin C
GI	Gastrointestinal
GRAS	Generally Recognised as Safe
GST	Glutathione S-transferases
HAART	Highly Active Antiretroviral Therapy
HBSS	Hanks' Balanced Salt Solution
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
IBU	Ibuprofen
IC_{50}	50% inhibitory concentration
IPA	isopropyl alcohol
IPEC	International Pharmaceutical Excipients Council
kg	kilogram
LA	Long acting
LPV	Lopinavir
MDCK	Madin-Darby Canine Kidney
MDCK-MDR1	MDCK transfected with MDR1 gene
MDR	Multidrug resistance
MEC	Minimum Effective Concentration
mg	Milligram
min	Minutes
mM	Millimolar
mRNA	messenger RNA
MRP	Multidrug resistance-associated proteins

MS	Mass Spectrometry
NAT	N-acetyltransferase
ng	Nanograms
NHS	National Health Service
NNRTI	Non-nucleotide reverse transcriptase inhibitors
NR	Nuclear Receptor
NRTI	Nucleotide reverse transcriptase inhibitors
OAT	Organic anionic transporter
OATP	Organic anion-transporting polypeptides
OCT	Organic cationic transporter
OI	Opportunistic infections
p	P Value
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
P_{app}	Apparent permeability
PBPK	Physiologically based pharmacokinetic
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PD	Pharmacodynamics
PI	Protease inhibitors
PK	Pharmacokinetics
PrEP	Pre-exposure prophylaxis
PXR	Pregnane X receptor
QC	Quality Control
QND	Quinidine
RED	Rapid Equilibrium Dialysis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT	Room Temperature
RT-PCR	Real-time Polymerase Chain Reaction
RTV	Ritonavir
SLC	Solute carrier

SNP	Single nucleotide polymorphisms
SPSS	Statistical Package for the Social Sciences
SSL	Sodium salicylate
SULT	Sulfotransferase
SVP	Sodium valproate
TAM	Thymidine analogue mutations
TB	Tuberculosis
TDF	Tenofovir disoproxil fumarate
TEER	Trans-epithelial electrical resistance
TPMT	Thiopurine S-methyl transferase
UGT	UDP-glucuronosyltransferase
UK	United Kingdom
USA	United States of America
UV	Ultra Violet
V_D	Volume of Distribution
WHO	World Health Organisation

Publications

Rohan Gurjar, Christina Chan, Paul Curley, Joanne Sharp, Justin Chiong, Steve Rannard, Marco Siccardi, Andrew Owen. 2018. "Inhibitory effects of commonly used excipients on P-glycoprotein in vitro." *Mol Pharm*. 2018 Nov 5;15(11):4835-4842. doi: 10.1021/acs.molpharmaceut.8b00482

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Communications

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General Abstract

Antiretroviral therapy is challenging due to the drug-drug interactions (DDIs) arising from the co-medications administered to treat co-morbidities and opportunistic infections. Drugs causing induction/inhibition of enzymes and transporters, or displacement from plasma proteins can alter drug pharmacokinetics (PK) and result in treatment failure due to elevated toxicities or suboptimal efficacy. Furthermore, inter-individual differences conferred by genetic variability responsible for modulating antiretroviral PK complicates treatment. Favourable PK is of paramount importance for successful treatment of any disease. Excipients are frequently used in drug formulations. These excipients not only transform the PK of drugs, but also exert biological effects in the body. These properties may be exploitable to modify PK for optimal outcomes. Evidently, investigations into the pharmacological properties of drugs and excipients are imperative for designing rational formulations and therapies to treat HIV. Knowledge about the effect of genetic polymorphisms on drugs will aid in vigilance of disparate outcomes and help personalising treatment.

The NEAT 001 / ANRS 143 was conducted to analyse the efficacy of a novel NRTI-sparing dual combination of darunavir/ritonavir and raltegravir against a standard-of-care triple therapy of darunavir/ritonavir and tenofovir/emtricitabine in HIV-infected antiretroviral naïve subjects. The single nucleotide polymorphisms (SNPs) that have known to have a clinical effect in previous literature were chosen and analysed for their influence on the drugs administered in this study, described in Chapter 2 and 3. In Chapter 4, the plasma protein binding and displacement of protease inhibitors – darunavir, atazanavir, lopinavir and ritonavir – were studied using Rapid Equilibrium Dialysis for their putative implications for DDIs. In Chapter 5, the effects of 25 commonly used excipients were analysed for their effects on P-glycoprotein (P-gp) by assessing the change in the cellular accumulation of a P-gp substrate digoxin in cells over-expressing P-gp. There is little data on the transport of the anti-tuberculosis agent linezolid which is frequently co-administered with antiretroviral drugs. Linezolid was assessed for transport by P-gp, MRP and the BCRP in Chapter 6.

Darunavir and ritonavir plasma levels were significantly lower in patients receiving raltegravir compared to those receiving tenofovir/emtricitabine, suggesting DDIs. *SLCO1B1* 521T>C (rs4149056) and *SLCO3A1* G>A (rs4294800) were associated with higher darunavir and ritonavir plasma concentrations, respectively. It was seen that the protease inhibitors bind to both AAG and albumin, suggesting a compensatory mechanism between the plasma proteins. P-gp inhibition by excipients was demonstrated by the following order of effects: Cremophor EL > Vit-E-PEG > Brij 58 > Tween 80 > NaCMC > Tween 20 > CTAB > Solutol HS 15 > AOT. Linezolid was found to be a substrate for P-gp, MRP and the BCRP and the results suggested involvement of additional transporters in linezolid disposition.

These findings not only corroborate previously published findings, but also presents novel findings that throw light on the mechanism of genetic variability, plasma protein displacement and induction/inhibition of transporters giving rise to DDIs.

Chapter 1

General Introduction

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1.1 Human Immunodeficiency Virus

In the early 1980s, clinical signs and symptoms of severe immunodeficiency were first observed in the United States, and then across the world; later to be known as Acquired Immunodeficiency Syndrome (AIDS).[1,2] This epidemic forced the scientific and medical communities world-wide to pool resources and investigate this disease. In 1983, French clinicians successfully isolated the human immunodeficiency virus (HIV-1) from a patient with AIDS.[3] This allowed researchers to gain knowledge about the nature of HIV/AIDS and to ultimately develop antiretroviral (ARV) drugs and strategies for treatment of individuals infected with HIV.[4]

In 2017, there were 36.9 million people living with HIV, with 1.8 million new infections; an 11% decline in new infections compared to 2010. A total of 940,000 deaths due to AIDS-related causes were recorded, representing a 48% decline when compared to 2005. These success have been attributed to antiretroviral therapy (ART), as well as treatment strategies that complement ART.[5] Nevertheless, factors such as adherence to ART, ART costs, suboptimal efficacy of ARVs, adverse effects, viral resistance to ARVs, and genetic variability between HIV-infected patients pose a challenge to the effective management of HIV/AIDS by ART.[6]

1.2 HIV Structure and Life Cycle

HIV is a Lentivirus, under the family of Retroviridae, subfamily Orthoretrovirinae. They are classified into two types, namely: HIV type 1 (HIV-1) and HIV type 2 (HIV-2) depending on their viral antigens.[7] The HIV virion is round and approximately 100 nm in size (**Figure. 1.1**). It has an outer envelope containing spikes of gp120 surface

proteins, anchored by gp41 transmembrane proteins.[8] The inner core consists of a capsid protein p24 containing two copies of a single-stranded genomic ribonucleic acid (RNA), reverse transcriptase, protease and integrase enzymes.[9]

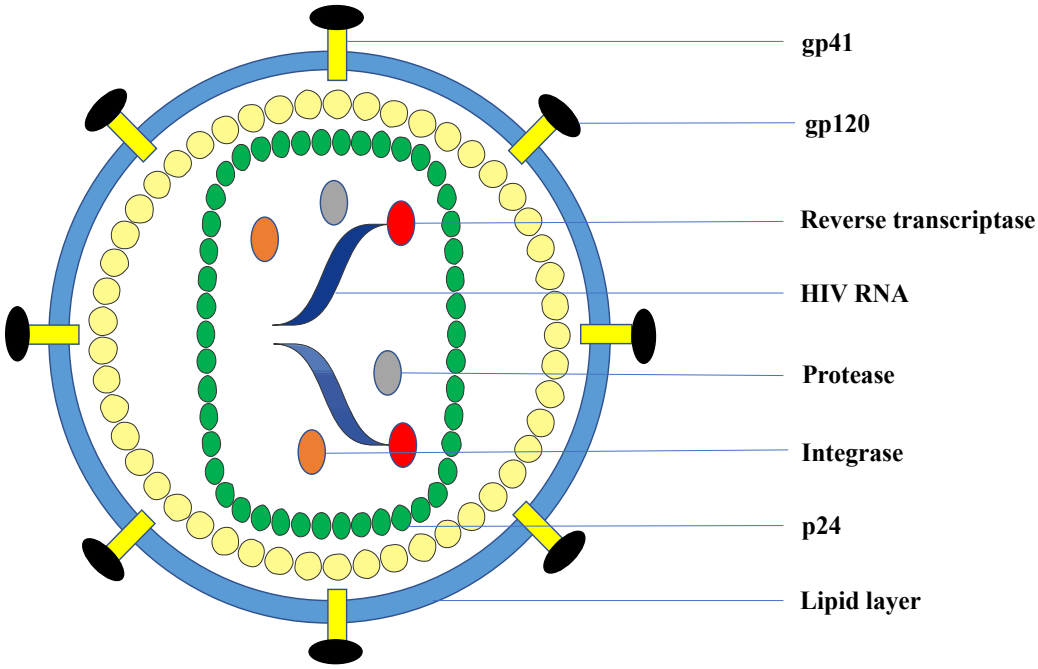

Figure 1.1. Structure of a HIV virion[7] containing surface proteins gp41 and gp120 and a p24 capsid enclosing HIV RNA along with reverse transcriptase, protease and integrase enzymes.

As shown in **Figure 1.2** HIV targets $CD4^+$ T lymphocytes and macrophages by attaching to the CD4 antigen via gp120,[10] with the help of co-receptors such as CXCR4 and CCR5 (Step 1).[11] Once bound to the cell, a series of conformational changes take place resulting in detachment of gp120 and exposure of gp41, initiating the fusion of $CD4^+$ and HIV (Step 2).[9] HIV enters the cell cytoplasm and sheds its

outer protein coat, exposing the core contents of the virion.[12] HIV-1 reverse transcriptase is activated and it transcribes the RNA into a deoxyribonucleic acid (DNA) (Step 3).[13] The DNA, along with the proteins enter the nuclear membrane of the host cell and gets integrated into the cellular genome of the cell with the help of integrase (Step 4).[14] DNA becomes a provirus by transcription (Step 5). The protease enzymes cleaves the Gag and Gag-Pol polyprotein precursors on the provirus to produce messenger RNAs (mRNA) of HIV and proteins, that are released into the systemic circulation (Step 6).[15]

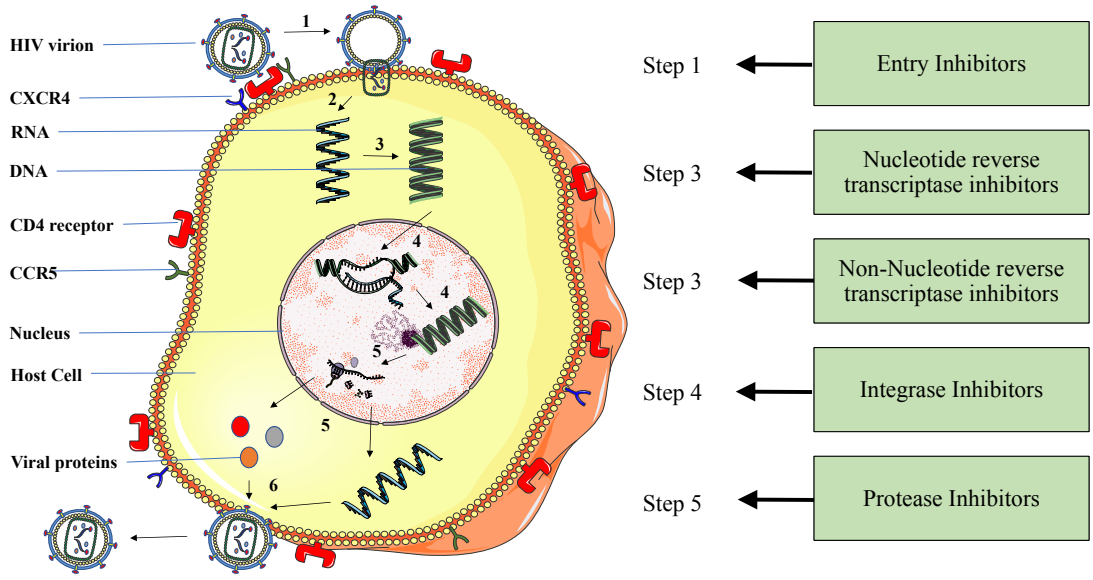

Figure 1.2. Life Cycle of HIV describing the entry of HIV into the cell, reverse transcription, integration into host genome and production of mature HIV virions. The steps at which antiretroviral drugs are described.

1.3 Opportunistic Infections associated with AIDS

The decrease in the CD4 cell count dysregulates the immune system and renders the body susceptible to opportunistic infections (OI). OIs were the greatest cause of morbidity and death in patients suffering from AIDS.[16] Most common OIs are pneumonia, candidiasis, herpes zoster and tuberculosis (TB). With the development of ARVs that have greater efficacy, less toxicity and are well-studied for potential drug-drug interactions (DDIs), the burden of OIs have reduced.[17] Due to effective disease management, more HIV-infected patients currently die from non-AIDS related illness than OIs.[18]

HIV is the strongest risk factor contributing to the resurgence of TB. The risk of developing TB is estimated to be between 16-27 times greater in people infected with HIV than among those without HIV infection. In 2015, there were an estimated 10.4 million cases of TB disease globally, including 1.2 million [11%] among people infected with HIV. A total of 57% of TB cases among people with HIV were not diagnosed or treated, resulting in 390,000 TB-related deaths among people living with HIV in 2015.[19] Bassett *et al*. showed that nearly 20% of patients starting ART in Durban had undiagnosed pulmonary TB.[20] Similarly, Antwal *et al*. demonstrated a Positive Predictive Value (PPV) of 78.8% and 80.6% for the detection of HIV in patients with TB and herpes zoster, who attended a HIV referral clinic.[21]

Treatment of TB along with HIV requires multidrug therapies which increase the risk of DDIs. Mesfin *et al*. demonstrated an increase in the risk of having multidrug resistant-TB (MDR-TB) in patients who are infected with HIV.[22] MDR-TB has exhausted the inventory of effective drugs, necessitating discovery of novel agents and formulations.[23]

1.4 Antiretroviral Drugs

ARVs have been successful in reducing HIV viral load in the blood and tissue of HIV-infected patients, by suppressing viral replication. They have not only reduced the morbidity and mortality of HIV-infected patients, but have also curbed the spread of the virus.[24] Reduction in HIV viral load leads to the restoration of $CD4^+$ cell count, responsible for restoring immunity in patients.[25] Although the treatment of HIV-infected patients is complicated, extensive research for over two decades has made it possible for a HIV-positive individual to have a life expectancy approaching that of healthy individuals.[26] ARVs are designed on the basis of the HIV life cycle by inhibiting processes involved in replication (**Figure. 1.2**).

1.4.1 Entry Inhibitors

Entry inhibitors act by blocking the interactions between gp120-CD4, gp120-co-receptor or gp41-mediated membrane fusion required for the entry of HIV into cells.[27] Enfuvirtide, a synthetic peptide that blocks the fusion of HIV1-gp41, was the first entry inhibitor approved for treatment of HIV patients.[28] Similarly, maraviroc is a CCR5 (co-receptor) antagonist that blocks the entry of HIV and is effective in treatment.[29] Other integrase inhibitors such as ibalizumab and fostemsavir are under investigation for possible use in ART.[30,31]

1.4.2 Nucleotide Reverse-Transcriptase Inhibitors (NRTIs)

After entering the host cell, the HIV genome undergoes reverse transcription from RNA to DNA which is catalysed by the reverse transcriptase enzyme. NRTIs are nucleoside analogues that lack the 3′–hydroxyl moiety that are incorporated into DNA by reverse transcriptase. The lack of 3′–hydroxyl moiety prevents the addition of further nucleotides, therefore ending chain extension by competitive inhibition.[32] Zidovudine was the first ARV to be approved for treatment in 1987 to treat HIV.[33] NRTIs are overwhelmingly used as a backbone in ART. Due to the low cost and efficacy, it is likely that NRTIs will play a significant role in the treatment and prevention of HIV.[34] However, concerns about cross-resistance and long term safety has raised interest in designing NRTI-sparing therapies.[35,36]

1.4.3 Non-Nucleotide Reverse-Transcriptase Inhibitors (NNRTIs)

Unlike the NRTIs, NNRTIs non-competitively inhibit HIV reverse transcriptase. The HIV reverse transcriptase enzyme is a part of the HIV virion (**Figure. 1.1**) and is not found in host cells, making it an ideal target to inhibit HIV replication without affecting other physiological processes.[37] The NNRTIs bind to a single position on the p66 subunit of the HIV reverse transcriptase called the NNRTI binding pocket. This inactivates the reverse transcriptase and inhibits the HIV replication cycle.[38] Based on their propensity for resistance, they are categorised into two generations. The first generation NNRTIs, such as nevirapine, delavirdine, and efavirenz, are drugs with low genetic barrier to resistance, and a single mutation is enough to render them ineffective. In contrast, the second generation NNRTIs, such as etravirine and

rilpivirine, have a greater barrier to resistance.[39] A new generation of drugs that are predicted to have fewer adverse effects and high barrier to resistance are also under development.[40]

1.4.4 Protease Inhibitors (PIs)

HIV protease plays an important role in the maturation of the HIV virus. It cleaves Gag and Gag-Pol polyproteins in the HIV virus at nine processing sites to produce mature proteins.[41] PIs bind to the protease with high affinity and block the binding of substrates needed in the HIV replication cycle.[42] Saquinavir was the first PI to be approved for treatment of HIV in 1995. The first-generation PIs such as saquinavir, fosamprenavir and indinavir had limited efficacy due to low bioavailability and resistance. This led to the development of second-generation PIs, such as darunavir and atazanavir, which are widely used for treating HIV.[43]

Most PIs have unfavourable PK due to short plasma elimination half-lives and variable bioavailability. The PK of PIs are enhanced by co-administrating agents, such as ritonavir and cobicistat, that inhibit CYP3A enzyme and P-glycoprotein (P-gp), resulting in increased absorption and reduced elimination.[44]

A new class of inhibitors called the maturation inhibitors are currently under investigation as a potential strategy to treat HIV.[45] These inhibitors act on protease, but are distinct from PIs in their mechanism of actions. Maturation inhibitors inhibit the final Gag processing step in which p25 is cleaved to p24 and SP1, rendering the HIV immature and non-infectious.[46]

1.4.5 Integrase Inhibitors

Integrase inhibitors block the integration of HIV-DNA into the host genome by binding to an enzyme present in the HIV virion called integrase. The integration of HIV is a multi-step process and provides opportunities to develop inhibitors with multiple mechanisms of action.[47] Raltegravir, developed by Merck and Co., was the first integrase inhibitor to be approved by Food and Drug Administration (FDA) for the treatment of HIV in 2007. Recently, dolutegravir and elvitegravir have been approved for their use against HIV.[48]

1.5 Highly-Active Antiretroviral Therapy (HAART)

One of the biggest challenges of monotherapy for the treatment of HIV was the emergence of resistance to the ARVs, resulting in treatment failure.[49] In the early 1990s, researchers experimented with treatments that included combinations of two or more ARVs and it was discovered that this resulted in greater success in inhibiting HIV replication, increasing CD4 counts and reducing AIDS-related illnesses, compared to treatment by monotherapies.[50,51,52] They significantly reduced the incidence of resistance and lowered treatment failure.[53] This strategy was named HAART.

ARVs that had similar dosing intervals were combined into a single pill to facilitate administration; called fixed-dose combinations (FDC). Combivir was the first FDC developed by GlaxoSmithKline Ltd in 1997. It contained 300 mg of atazanavir, 150 mg of lamivudine and 300 mg zidovudine, an approved backbone for treatment of HIV popular at that time.[54] Since then, the FDA has approved a number FDCs such

as Trizivir (abacavir, zidovudine and lamivudine) and Atripla (efavirenz, emtricitabine and Tenofovir disoproxil fumarate; TDF) for the treatment of HIV.[55]

1.6 Pre-Exposure Prophylaxis (PrEP)

ARVs are not only used to treat individuals infected with HIV, but are also used to reduce the spread of the disease in populations. Pre-exposure prophylaxis **(**PrEP) involves the administration of ARVs to non-infected individuals in high-risk environments. The ARVs in the body restricts HIV from entering and replicating, and does not allow the virus to establish itself within the body.[56] The effectiveness of PrEP was first demonstrated in the CAPRISA 004 trial in which the likelihood of HIV acquisition in women taking tenofovir vaginal gel was 39% lower than women taking placebo gel.[57] Soon thereafter, various drugs were tested for their efficacy in PrEP. In 2012, orally administered Truvada® (Tenofovir 300 mg/emtricitabine 200 mg) was approved by FDA, and is currently the only FDC available for PrEP.[58]

1.7 Antiretroviral Pharmacokinetics

Pharmacokinetics (PK) is defined as "the study of movement of drugs in the body in terms of absorption, distribution, metabolism and excretion".[59] ARVs are mostly administered orally, except for enfuvirtide, which is administered subcutaneously twice daily.[60] Drug PK is measured by calculating the C_{max}, C_{min} and area under the curve (AUC), which are derived by measuring the drug concentration in blood plasma at specific intervals after dosing. This is summarised in **Figure. 1.3**.

i. C_{max} – Highest drug concentration observed after oral administration

ii. C_{min} – Lowest drug concentration observed after oral administration

iii. AUC – Total area under the curve when drug concentration against time

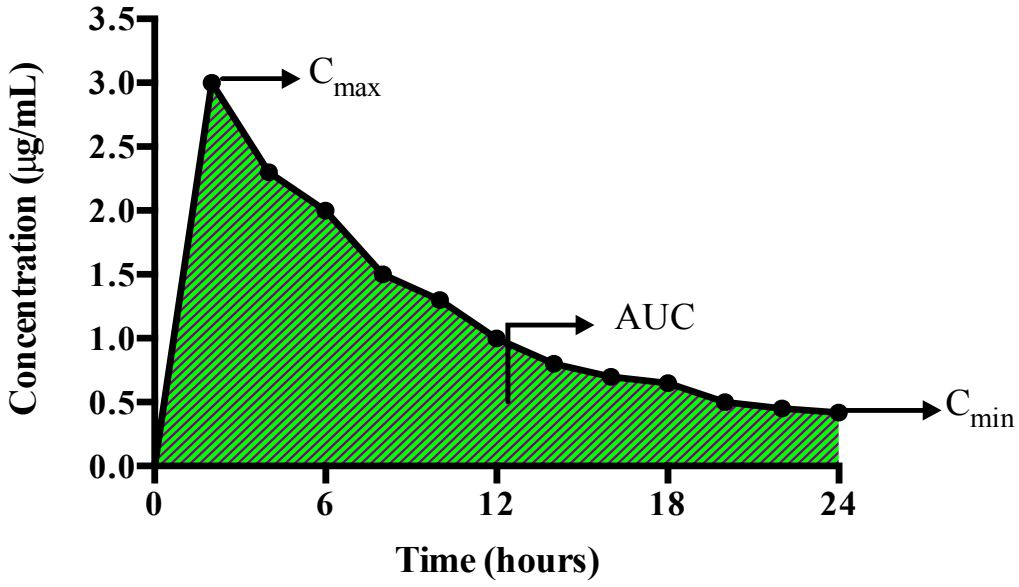

Figure 1.3. An example of a drug's pharmacokinetic curve, showing the C_{max}, C_{min} and AUC of the drug after oral dosing.

1.7.1 Absorption

Drug bioavailability is the fraction/percentage of an extravascularly administered dose that reaches the systemic circulation via absorption.[61] Absorption depends on a drugs' characteristics such as aqueous solubility, membrane permeability and stability.[62] Moreover, external factors of the gastrointestinal (GI) tract such as GI motility, efflux transporters, pH, the presence or absence of food, altered physiology

due to disease, as well as first-pass metabolism, each play a role in affecting drug bioavailability.[63] Apart from TDF, didanosine and efavirenz, most NRTIs and NNRTIs have good bioavalability.[64] By contrast, PIs have low bioavailability and are combined with ritonavir, a P-gp and CYP3A inhibitor, to increase their plasma exposure.[65]

1.7.2 Distribution

The apparent volume of distribution (V_D) of a drug is defined as "the volume of plasma needed to account of the total amount of drug present in the body", and is affected by pK_a, Log*P* and the degree of protein binding of the drug. The distribution of a drug determines the proportion of drug that reaches the tissues as well as the drug half-life.[66]

1.7.3 Metabolism

Metabolism is the major route of elimination for most drugs. Liver is the principle site for most of the metabolism. However, enzymes are also present in extra-hepatic sites such as kidney, lungs, skin, adrenal glands and intestinal mucosa.[67]

Phase I metabolism usually exposes hydrophilic groups on the drug via oxidation, reduction or hydrolysis, mostly by cytochrome P450 monooxygenase (CYP) enzymes.[68] Phase II metabolism involves conjugation reactions via glucuronidation, sulfation, acetylation, glutathione conjugation, methylation and amino acid conjugation. These are carried out by UDP-glucuronosyltransferases (UGTs),

sulfotransferases (SULTs), N-acetyltransferases (NATs), glutathione S-transferases (GSTs) and various methyltransferases (mainly thiopurine S-methyl transferase, TPMT; and catechol O-methyl transferase, COMT).[69] All NNRTIs[39], and PIs[70], are metabolised extensively by CYP enzymes. Integrase inhibitors are metabolised via the UGT1A1 pathways, occasionally coupled with CYP.[71]

1.7.4 Excretion

Xenobiotics and metabolites are excreted mainly through renal and biliary excretion. Small and polar compounds undergo filtration, secretion and reabsorption, and the unwanted products are excreted in the urine. The larger, lipophilic compounds are excreted through bile.[72]

1.8 Alternative Routes of Administration

Oral routes of administration at times can be inconvenient for patients with debilitating conditions. For example, a patient infected with oral *Candida* or suffering from esophagitis may have difficulty in swallowing a pill.[73] Moreover, oral administration has limitations, such as pill burden, first-pass metabolism, interference by GI contents, variable bioavailability or GI toxicity. This could lead to lack of adherence by patients, exacerbating resistance and contributing to treatment failure.[74] To obviate these shortcomings, novel drug delivery strategies are under investigation.

Long-acting (LA) formulations of ARVs have gained popularity in the treatment of HIV. Their administration is mostly intra-muscular, sub-cutaneous or via implants

such as vaginal rings.[75] Topical microbicides such as vaginal gels and rectal douches are also being investigated for PrEP.[76,77] However, these routes of administration pose special challenges due to differences in their PK as compared to oral administration.[75] For example, first pass metabolism is avoided by vaginal and rectal delivery, resulting in a higher bioavailability of drugs when compared to oral administration, requiring changes in dose administered.[78]

1.9 Antiretroviral Pharmacodynamics

AIDS is defined as "the most advanced stage of HIV infection where an infected individual's immune system is damaged and is exposed to OIs. It is characterised by either a CD4 count < 200 cells/mm^3 or by an AIDS defining illness such as TB, herpes zoster, non-Hodgkin lymphoma and Kaposi's sarcoma." As per the 2017 World Health Organization (WHO) guidelines, all countries are advised to follow the "treat all" policy, where any person diagnosed as HIV positive or as having AIDS should be initiated on ART within seven days.[79] The goals of ART are to reduce viral load below detectable levels (< 50 copies/mL) and to increase CD4 cell count in HIV-infected individuals. Inability to do so leads to treatment failure. (**Table 1.1**).[80]

Numerous factors need to be considered for the successful treatment of a HIV-infected individual with ART and are discussed below:

Table 1.1. Definition of clinical, immunological and virological failure according to the WHO[80]. Refer **Table 1.2** for Clinical Staging of HIV

Type of Failure	**WHO Definition**
Clinical	In adults, new or recurrent Stage 4 clinical event after six months of ART In children, new or recurrent Stage 3 or 4 clinical event after six months of ART
Immunological	In adults, CD4 count falls below baseline or persistently <100 cells/mm^3 In children, CD4 levels < 200 cells/mm^3
Virological	Plasma viral load above 1000 copies/mL on two consecutive measurements after three months

Table 1.2 WHO clinical staging of HIV disease in adults and children

Stage	Adults	Children
1	Asymptomatic	Asymptomatic
2	Moderate Weight Loss Respiratory Tract Infections Herpes Zoster	Hepatosplenomegaly Respiratory Tract Infections Herpes Zoster
3	Severe Weight Loss Chronic Diarrhoea Oral Candidiasis Pulmonary Tuberculosis Severe Bacterial Infections	Malnutrition Chronic Diarrhoea Oral Candidiasis Pulmonary Tuberculosis Severe Bacterial Infections
4	HIV Wasting Syndrome Pneumonia Herpes Simplex Oesophageal Candidiasis Extra-pulmonary Tuberculosis Kaposi Sarcoma HIV Encephalopathy	HIV Wasting Syndrome Pneumonia Herpes Simplex Oesophageal Candidiasis Extra-pulmonary Tuberculosis Kaposi Sarcoma HIV Encephalopathy

1.9.1 Safety and Toxicity of Antiretroviral Drugs

Drug toxicity is a major concern for the successful treatment of HIV patients with HAART and is one of the most common cause of changing treatment regimens.[81] NRTIs inhibit human DNA polymerases, resulting in the production of dysfunctional mitochondria and the generation of reactive oxygen species (ROS). ROS damage proteins, lipids and DNA, giving rise to mitochondrial toxicity, which is characterised by myopathy, neuropathy, lipoatrophy and lactic acidosis.[82] The symptoms can be severe, and require change in treatment.[83] To avoid these complications, new strategies of NRTI-sparing regimens are under investigation.[35] NNRTIs have a lower incidence of adverse events compared to the NRTIs. They are metabolised by the liver and can cause rash, hepatotoxicity and Stevens-Johnson syndrome. Efavirenz is associated with CNS toxicities such as mood swings, insomnia and nightmares. However, these symptoms can disappear over time.[84]

Initiation of treatment with PIs is often accompanied with GI symptoms such as abdominal pain, nausea, vomiting and diarrhoea, but often resolve quickly.[85,86] Long-term use of PIs is associated with dyslipidaemia due to increased hepatic secretion of very low-density lipoprotein giving rise to insulin resistance[87] and cardiovascular diseases.[88] Entry inhibitors such as enfuvirtide and maraviroc can give rise to hypersensitivity reactions and hepatotoxicity; however, incidences are rare.[89] No severe adverse effects have been reported with the integrase inhibitor raltegravir.[86]

1.9.2 Antiretroviral Resistance

Antimicrobial resistance is the ability of a microorganism to stop an antimicrobial from working against it. It is a growing global threat that necessitates efforts to ensure prevention.[90] The emergence of HIV resistance to ARVs reduces treatment options due to the limited number of ARVs available.[91] Drug resistance can either be transmitted, where a drug-resistant strain is transmitted from one person to another, or acquired by suboptimal treatment. Strong correlations between lack of adherence to drugs and resistance have been observed.[92] A fall in the drug plasma concentration below the minimum effective concentration (MEC) allows the development of a resistant virus, which renders the ARV ineffective.[93] In some cases, resistance to one drug results in resistance to other drugs; a phenomenon known as cross-resistance.[94] For example, the G118R substitution results in a statistically similar resistance to dolutegravir, raltegravir and elvitegravir.[95]

Resistance to NRTIs can occur either by recognising and differentiating the NRTI from dNTP, or by primer unblocking mutations that lead to phosphorylytic excision of the NRTI from the viral DNA. The primer unblocking mutations are called thymidine analogue mutations (TAMs).[96] Mutations in response to NNRTIs have a high potential for cross-resistance since the mutation sites encode amino acids that are adjacent to each other within the NNRTI-binding pocket. Additionally, the genetic barrier to NNRTI resistance is low, and a single mutation is sufficient to confer resistance.[97] Resistance to PIs occurs due to accumulation of amino acid substitutions in the HIV protease that prevent PIs from inhibiting it.[98] A significant amount of cross-resistance is seen in PIs since they all bind to a similar site on the HIV protease.[99] Integrase inhibitor resistance occurs due to mutations in the integrase

enzyme that make the integrase enzyme less susceptible to inhibition. They are characterised by a low genetic barrier and major cross-resistance.[100] Enfuvirtide, a fusion inhibitor, has a low genetic barrier resulting in rapid onset of mutations resulting in resistance.[96]

The WHO has developed a global action plan for HIV drug resistance. The strategies include interventions for prevention and response to resistance, monitoring and surveillance of resistance, research and innovations to minimize drug resistance, strengthening laboratory capacities to monitor resistance and ensuring proper governance to support action on drug resistance strategies.[101]

1.9.3 Efficacy of HAART

The efficacy of ARVs depends on adverse events, adherence to treatment regimens, barrier to HIV resistance, cost and the ability to reduce HIV viral load and increase CD4 count. These factors are taken into consideration while formulating guidelines for HAART.[102]

With the approval of zidovudine and didanosine, HIV-infected individuals were treated with NRTI-monotherapy, which improved patient survival and halted disease progression. Zidovudine monotherapy reduced the risk of maternal-infant HIV transmission when given orally during pregnancy.[103] Subsequently, it was discovered that NRTI-dual therapy had greater benefits to the patients than monotherapy, but exhibited greater toxicities. They also failed to halt CD4 decline, rendering patients susceptible to OIs.[104,105] With the invention of PIs, it was found that a combination of PI + 2NRTIs exhibited remarkable benefits in terms of viral load reduction and

increase in CD4 count.[106,107] Similar treatment success was observed when an NNRTI nevirapine was administered with 2 NRTIs.[108] A triple NRTI regimen was proposed to obviate the additional adverse events of PIs such as insulin resistance and hyperlipidaemia; however, this combination was inferior in terms of potency.[109] These findings confirmed a place for 2 NRTIs as a backbone for first-line therapy to treat HIV-infected individuals. Recently, attempts to design NRTI-sparing regimens, to avoid the toxicities of NRTI have shown success. The clinical trial NEAT001 confirmed that a NRTI-sparing combination of darunavir boosted with ritonavir along with raltegravir, was non-inferior in efficacy compared to ritonavir-boosted darunavir with tenofovir and emtricitabine.[110] Notwithstanding the low genetic barrier, a first-line therapy with a NNRTI has been adopted by many countries, and has been successful in treating HIV. Etravirine, a second-generation NNRTI devoid of major toxicities and cross-resistance, has added to the interest in this class of ARVs.[111]

When administered on their own, PIs have a low bioavailability and need to be dosed multiple times a day. This gives rise to complications such as food-dependent dosing, a high pill burden resulting in lack of adherence, inter- and intra-patient variability, high first-pass metabolism, DDIs, variable half-life, high cost and low penetration of the PI into HIV sanctuary sites. On co-administration with ritonavir, a P-gp and CYP3A4 inhibitor, PIs have higher bioavailability due to increased tissue penetration and decreased PI clearance. This lowered the quantity and frequency of doses.[112,65] It also conferred PIs with a high genetic barrier.[113]

Guidelines provided by the FDA and the European Medicines Agency (EMA) suggest raltegravir with a tenofovir/emtricitabine backbone as a first-line treatment for HIV-infected individuals.[114] Raltegravir displays good tolerability and limited DDIs due to

its metabolism via the UGT1A1 pathway. Elvitegravir and dolutegravir are also promising drugs since they have similar properties to raltegravir.[115]

Enfuvirtide was the first fusion inhibitor to be approved in 2003 following a Phase III trial, which demonstrated effectiveness against HIV when combined with a backbone regimen.[60] It is an oligopeptide and is administered subcutaneously, but is also associated with pain at the site of injection. Since it has a different mechanism of action than other ARVs, enfuvirtide is effective against HIV with multiple resistance mutations to other classes and is used as a salvage therapy.[116] The efficacy of maraviroc has been established in the MOTIVATE and MERIT studies.[117] Maraviroc acts by binding to the CCR5 receptor, blocking HIV entry into the cell. However, maraviroc does not block CXCR4, an alternative route for HIV to enter a cell.

1.10 Drug-Drug Interactions with Antiretroviral Drugs

DDIs can lead to suboptimal drug exposure, or conversely may increase drug exposure, giving rise to toxicity and adverse drug reactions. They can result in treatment failure and in some cases elicit significant harm to patients.[118,119] A HIV-infected individual can often present with additional ailments such as TB, malaria, hepatitis, or non-communicable indications which require treatment.[79] Moreover, with the increase in HIV patients over 50 years of age, chronic conditions associated with ageing requiring cardiovascular drugs, chemotherapy, antihypertensive and lipid lowering drugs are becoming more common.[120] Co-administration of drugs required for these illnesses increase the likelihood of DDIs with ART. DDIs alter the absorption, distribution, metabolism and excretion of drugs.[121] The mechanisms

giving rise to DDIs involve induction or inhibition of metabolic enzymes, induction or inhibition of drug transporters or plasma protein displacement, resulting in altered PK.[122] Interactions are also shown to happen at the intra-cellular level involving influx transporter on the CD4+ cells. For example, Liptrott *et al.* demonstrated a 39% and 73% reduction in intra-cellular nevirapine in the presence of tenofovir in CD4+ cells and monocyte-derived macrophages. Similarly, nevirapine caused a 57% decrease in tenofovir accumulation.[123]

These mechanisms are discussed in greater detail below.

1.10.1 Induction/Inhibition of Metabolic Enzymes

DDIs involving metabolic enzymes occur either due to the inhibition, or induction of enzyme expression and activity.[124] The discovery of CYPs and their importance in metabolism of drugs played an important role in understanding DDIs.[125] There are more than 50 subtypes of CYP, which collectively metabolise 90 percent of available drugs, and are present in liver, lungs, intestine, placenta and kidneys.[126]

Inhibition of CYP enzymes can slow the metabolism of substrate drugs, elevating serum levels that can result in toxicity and adverse events, especially for drugs with narrow therapeutic index. CYP inhibition can either occur reversibly or irreversibly (**Figure. 1.4**).

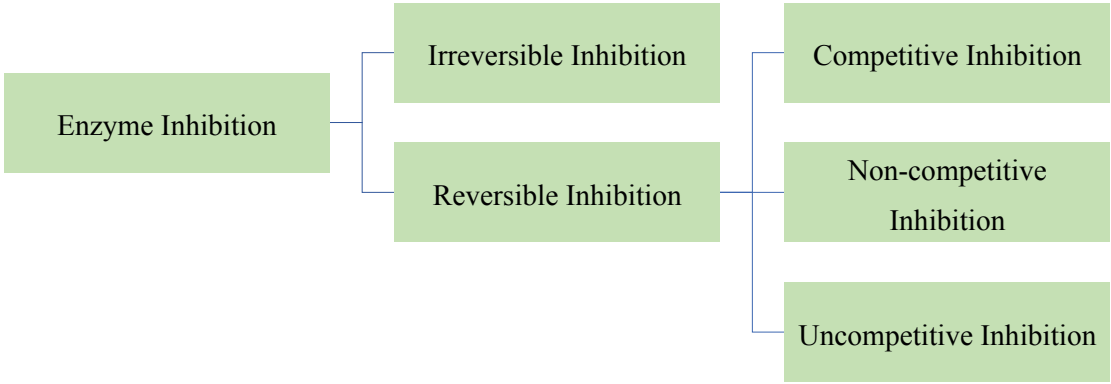

Figure 1.4. Types of enzyme inhibition

Irreversible inhibition occurs via formation of a metabolite complex to a protein or heme of the CYP enzyme and the effect is long-lasting. Reversible inhibition can be competitive, non-competitive, uncompetitive or mixed (**Figure. 1.5**).[127] In competitive reversible inhibition, drugs compete to bind to the same site on the CYP enzyme, whereas in non-competitive, the binding site is different. In uncompetitive reversible inhibition, the inhibitor binds to the enzyme-substrate complex.[124]

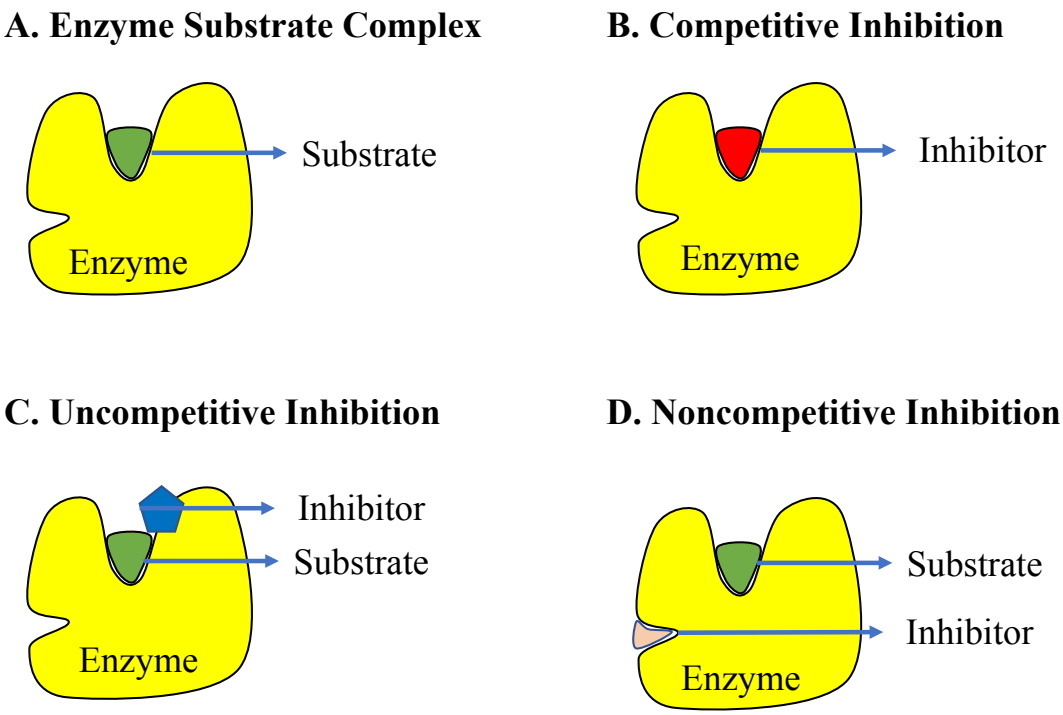

Figure 1.5. Types of reversible enzyme inhibition (B) Competitive Inhibition (C) Uncompetitive inhibition (D) Non-competitive inhibition

Induction of CYP enzyme expression, on the other hand, increases the rate of metabolism of enzyme substrates, resulting in increased clearance and a suboptimal effect of that drug. For example, rifampicin is a potent inducer of CYP3A5 and leads to significant decrease in exposure of CYP3A4 substrates.[128] Induction is a longer process compared to inhibition as it takes time to reach higher steady state enzyme levels. The mechanism of CYP enzyme induction can involve aryl hydrocarbon receptor (AhR), nuclear receptor pregnane X receptor (PXR) and constitutive androstane receptor (CAR).[129] AhR is expressed widely in human tissues and regulates the expression of the CYP1 and CYP2 family of enzymes. Upon binding to an agonist, it localises in the nucleus and forms a AhR/AhR nuclear translocator which binds to the DNA.[130] PXR is mostly localised in the liver and small intestine

and largely affects CYP3A4, with some influence on CYP2A, CYP2B and CYP2C. In addition to drugs, PXR is activated by endogenous substances like steroids and bile acids and is responsible for variability in CYP3A4. Recent studies have shown that miRNAs (microRNAs) such as miRNA-148a influence PXR and CYP3A4.[131] CAR is expressed in the liver and kidney and is activated by ligand-dependent and ligand-independent mechanisms. CAR induces CYP2B6, CYP3A4, CYP2Cs, CYP2A6, CYP1A1 and CYP1A2, which contribute to the metabolism of 75 percent of all drugs prescribed.[132] CYPs are responsible for the metabolism of most of the NNRTIs, PIs and integrase inhibitors.[133] NNRTIs and PIs induce and inhibit certain CYP enzymes. For example, darunavir is a substrate of CYP3A4 and is reported to inhibit CYP3A4 and induce CYP2C9.102

In addition to metabolism by CYP enzymes, integrase inhibitors dolutegravir and elvitegravir are also metabolised by UGT1A1 enzymes. Raltegravir, on the other hand, is not metabolised by CYP. Some have argued that this difference in disposition compared to other classes of drugs minimises the potential for DDIs and makes raltegravir more suitable for combination therapy.[134] UGTs are a superfamily of enzymes that metabolise endogenous substances (steroids, bilirubin and vitamins) and exogenous substances like carcinogens, pollutants and drugs.[135] UGT1A1 is induced by ritonavir and is inhibited by both atazanavir and efavirenz. However, no dose adjustments to integrase inhibitors are necessary when co-administered with the UGT1A1 inducers.102,[136]

1.10.2 Induction/Inhibition of Drug Transporters

The disposition of ARVs involve transporters such as adenosine triphosphate (ATP) – binding cassette (ABC) transporters and solute carrier (SLC) transporters. The induction or inhibition of these transporters caused by a co-administered drug leads to DDIs, and can complicate ART.[137] DDIs involving drug transporters occur during intestinal absorption, hepatic elimination and/or renal excretion. Transporter DDIs can sometimes also affect drug penetration into sanctuary sites such as the CNS, genital organs or lymphocytes.[138]

The ABC superfamily is the largest and most ubiquitously expressed transporter family in the human body and are involved in the transport of substances using ATP hydrolysis.[139] P-gp (MDR1; ABCB1) is an efflux transporter expressed in the liver, kidneys, blood-brain barrier (BBB), blood-placenta barrier and blood-intestinal barrier.[140] It has a huge number of drug substrates, including chemotherapeutic drugs, immunosuppressant drugs, antiepileptic drugs and ARVs. P-gp is responsible for the transport of all PIs and other ARVs such as TDF, zidovudine, abacavir, maraviroc, raltegravir and dolutegravir.[141] It is inhibited by PIs such as ritonavir, lopinavir and nelfinavir as well as NNRTIs such as efavirenz and delavirdine. P-gp activity is enhanced by long-term exposure to ARVs including ritonavir, lopinavir, atazanavir, efavirenz, lamivudine.[138] One of the biggest challenges in treating HIV patients co-infected with TB is the induction of P-gp and CYPs by rifampicin, leading to reduced bioavailability of ARVs.[142]

The multidrug resistance-associated protein (MRP) family has 13 members, of which MRPs 1—9 are involved in drug transport.[143] Many PIs such as ritonavir, lopinavir, atazanavir and saquinavir, are substrates of MRP1 and MRP2.[144,129] in addition to

MRP1 and MRP2, tenofovir is also a substrate of MRP4 an MRP5.[145] Delavirdine, efavirenz and emtricitabine inhibit MRP1-3, while ritonavir and nelfinavir has been reported to increase MRP2 expression.[141] Breast cancer resistance protein (BCRP/ABCG2) is involved with the development of resistance to zidovudine, lamivudine, didanosine and stamivudine and is inhibited by atazanavir, ritonavir, lopinavir, delavirdine and efavirenz.[146,147]

SLC transporters are involved with the influx and efflux of a wide range of nutrients, drugs and xenobiotics across biological membranes.[148] Organic anion-transporting polypeptides (OATPs) - members of the SLC family - are expressed in the liver, intestine, kidneys, lungs, testis and brain, and tend to be expressed on the basolateral side of cells.[149] Many PIs such as saquinavir, lopinavir and darunavir are substrates of OATPs. OATPs are also inhibited by atazanavir, indinavir, saquinavir, ritonavir and nelfinavir; induced by nelfinavir and ritonavir.[150] Organic anion transporters (OATs) and organic cation transporters (OCTs) are expressed mainly in the kidneys and liver. OAT4 transports NRTIs, while OAT1 and OAT3 are involved in uptake of tenofovir. PIs such as nelfinavir, ritonavir, saquinavir and indinavir inhibit OCT1 and OCT2.[138] Nucleoside transporters, such as concentrative transporters (CNT) and equilibrative transporters (ENT) are involved the transport of nucleoside analogues such as zidovudine, lamivudine and didanosine.[151]

1.10.3 Plasma Protein Displacement

Many drugs when absorbed and upon reaching the systemic circulation bind to circulating plasma proteins: mainly α_1- acid glycoprotein (AAG; also known as orosomucoid) and/or albumin; and to a lesser extent globulins and lipoproteins.[152] The percentage of the drug that remains unbound to plasma proteins is free to exert its biological effects or to be cleared from the body, whilst the protein-bound drug remains latent (**Figure. 1.6**). The presence of a concomitantly-administered drug with a higher affinity to plasma proteins can cause displacement and an increase in the unbound percentage of the victim drug, resulting in a DDI.[153] However, there is a current lack of consensus regarding the clinical relevance of plasma protein displacement.[154]

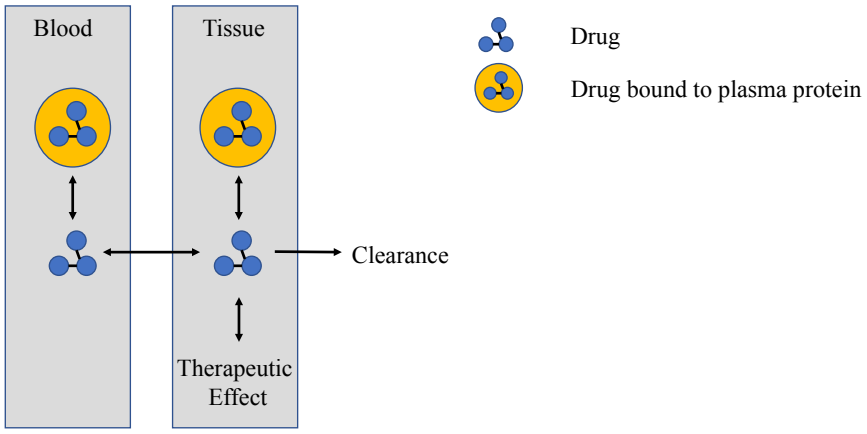

Figure 1.6. Role of plasma protein binding on drug pharmacokinetics and pharmacodynamics

1.11 Pharmacoenhancers

Ritonavir leads to extensive DDIs due to its inhibition of CYP3A4 and P-gp.[155] Such interactions exhibited by ritonavir have been exploited to reduce the metabolism of other PIs and has been termed pharmaco-enhancement or "boosting". Low-dose ritonavir increases absorption and decreases metabolism of co-administered PIs, resulting in a reduction in the required dose, which is beneficial for adherence and the barrier to resistance.[65] However, it cannot be administered to boost other classes of ARVs due to the risk of PI resistance resulting from low dose exposure of ritonavir.[156]

Cobicistat (COBI) is another pharmacokinetic enhancer and acts by inhibiting CYP3A enzymes, CYP2D6, P-gp, BCRP, OATP1B1 and OATP1B3. It has been reported to inhibit the metabolism of elvitegravir, atazanavir and darunavir.[157] A FDC containing elvitegravir/cobicistat/emtricitabine/TDF was approved by FDA in 2012 as a once-daily pill.[158] Since then, COBI has been approved for FDCs such as Rezolsta and Evotaz to boost darunavir and atazanavir, respectively.[159]

1.12 Pharmacogenetics in Antiretroviral Therapy

Pharmacogenetics is the discipline that analyses the genetic basis for inter-individual variation in the PK of drugs.[160] Individuals differ in genetic make-up, which affects the final products of transcription and translation. Genetic changes such as single nucleotide polymorphisms (SNPs), gene deletions and gene duplications may produce enzymes, transporters and plasma proteins that can differ in activity. This can potentially alters both drug PK and drug pharmacodynamics (PD).[161]

More than 2000 mutations have been identified in genes coding for CYP enzymes and the most important polymorphic CYPs are 1A2, 2D6, 2C9 and 2C19.[162] Similarly, polymorphisms in genes coding for transporters and plasma proteins have shown to influence the PK of drugs (**Table 1.3**).

Table 1.3. Influence of polymorphisms on protein expression and activity resulting in changes in drug pharmacokinetics

Protein	Polymorphism	Change in activity	Clinical Implications
CYP2B6	*CYP2B6* 516 G>T	Reduced expression	Higher efavirenz concentrations[163]
	CYP2B6 785 A>G	Increased expression	Higher efavirenz concentrations[164]
	CYP2B6 983 T>C	Reduced expression	Higher efavirenz concentrations[164]
CYP3A4	*CYP3A4*B1*	Reduced expression	Higher efavirenz concentrations[165]
	*CYP3A4*22*	Reduced expression	Higher lopinavir concentrations[166]
CYP3A5	*CYP3A5*3*	Increased expression	Lower atazanavir concentrations[167]
UGT1A1	*UGT1A1*36*	Reduced expression	Higher raltegravir concentrations[168]
PXR	*NR1I2* 63396 C>T	Higher CYP3A4 expression	Lower atazanavir concentrations[169]
CAR	*NR1I3* 540 C>T	Higher CYP3A4 expression	Lower efavirenz concentrations[170]
ABCB1	*ABCB1* 3435 C>T	Change in substrate specificity	Higher efavirenz concentration
	ABCB1 2677 G>T	Decreased expression	Lower atazanavir concentrations
MRP2	*ABCB2* 24 C>T	Decreased activity	Higher tenofovir clearance
MRP4	*ABCB4* 3463 A>G	Decreased activity	Higher tenofovir concentrations[171]
AAG	*ORM1*S*	Increased binding affinity	Increased telmisartan AUC[172]

1.13 Thesis Aims

The overall aim of the thesis was to investigate the DDIs that influence the treatment of HIV *in vitro* and *in vivo*. ARVs are given in combination, and are often co-administered with drugs to combat TB, herpes zoster, hepatitis, diabetes as well as anti-chemotherapeutic agents, which can each result in DDIs leading to toxicities or treatment failure.[79] In this thesis, research was focused not only on investigating pharmacogenetic associations with PK in a clinical cohort, but also to understand the mechanistic basis for potential DDIs.

Chapter 2 and **Chapter 3** describe the effects of genetic variability on drug plasma concentrations in patients enrolled in the NEAT001/ANRS143 trial. The primary objective of the trial was to test the efficacy of an NRTI-sparing regimen (darunavir/ritonavir + raltegravir), in comparison with an established standard-of-care regimen (darunavir/ritonavir + TDF + emtricitabine).[110] The polymorphisms that have been found to have clinically significant effects on other PIs were analysed for their effects on darunavir, ritonavir, raltegravir, tenofovir, and emtricitabine PK and DDIs.

The NEAT001 study showed an unexpected interaction between darunavir and raltegravir, which led to a decrease in darunavir plasma concentrations, and was associated with treatment failure.[110] In theory, the PK of darunavir and raltegravir in terms of induction and inhibition of enzymes and transporters are exclusive and a DDI was not expected. The possibility of the observed DDI resulting from darunavir displacement from plasma proteins when co-administered with raltegravir was therefore explored, and is described in **Chapter 4**.

The inertness of excipients has been challenged in recent years and studies have shown an impact upon CYP enzyme activity and PK.[173] In **Chapter 5**, the effect of 25 commonly used excipients on P-gp activity was assessed by measuring the change in cellular accumulation of digoxin (a model P-gp substrate) in Madin-Darby Canine Kidney transfected with the MDR1 gene (MDCK-MDR1).

Linezolid, an oxazolidinone antibiotic has been successful in treatment of a wide range of illnesses due to infections from organisms such as *Enterococcus faecalis, Staphylococcus aureus, Chlamydia pneumoniae, Haemophilus influenza,* and MDR-TB.[174] Anti-tubercular drugs are often co-administered with ARVs and can lead to complications such as DDIs and toxicities. A thorough investigation of the properties of ARVs as well as anti-tubercular drugs are necessary to design rational strategies for managing HIV and TB and predict treatment outcomes.[175] Currently, the pharmacokinetic profile of linezolid with regard to its transport is incomplete. Substrate recognition by P-gp, BCRP and MRP transporters was studied by measuring the transport of linezolid in the presence of P-gp, BCRP and MRP inhibitors, as described in **Chapter 6**.

Chapter 2

Pharmacogenetics of Ritonavir-Boosted Darunavir and Ritonavir in HIV-infected adults: a sub-study of NEAT001/ANRS143

Contents

2.1 Introduction

2.2 Methods

2.2.1 Study Design and Samples Collection

2.2.2 DNA Extraction and Genotyping

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2.3 Results

2.3.1 Patient Characteristics and Genotyping

2.3.2 Influence of SNPs on Darunavir Plasma Concentrations

2.3.3 Influence of SNPs on Ritonavir Plasma Concentrations

2.4 Discussion

2.1 Introduction

Darunavir is a HIV-1 PI and is effectively used for combating HIV in treatment experienced and treatment naïve patients.[176] Like all PIs, it is extensively metabolized in liver and the intestinal lumen by the CYP enzymes and has a high rate of elimination by first pass metabolism, resulting in low bioavailability.[177] To improve the bioavailability, darunavir is co-administered with pharmacoenhancers such as ritonavir or cobicistat.[178,179]

Ritonavir, a PI, was initially used in combination with other ARVs to combat HIV. However, adverse events such as diarrhoea, nausea, vomiting, abdominal pain and rash led to discontinuation of ritonavir for HIV treatment.[180] It was noticed that at low doses, ritonavir is a highly potent inhibitor of CYP3A4 and P-gp, and improved the bioavailability of PIs including darunavir, lopinavir and atazanavir.[155] For example, on co-administration with ritonavir, the bioavailability of darunavir increases from 37% to 82%.[181] This boosting results in a decrease in pill burden and frequency of dosing of PIs and helps to improve adherence.[182]

The POWER 1 and 2 studies demonstrated that darunavir/ritonavir 600/100 mg, administered twice daily, was most effective in controlling HIV with favourable safety, compared to other PIs.[183] Soon after, the ARTEMIS trial confirmed the efficacy of darunavir/ritonavir 800/100 mg, administered once daily.[184] Darunavir is well tolerated in pregnant women[185] and paediatric patients with HIV[186] and has a high genetic barrier to resistance.[187] These advantages have made darunavir the preferred PIs for treatment of HIV.[44] Traditionally, darunavir boosted with ritonavir has been administered along with a back-bone of 2 NRTIs.[35] However, NRTI-sparing regimens are now also being explored to mitigate some associated problems.[188,189]

A recurring challenge for the efficient treatment of HIV with darunavir is the inter-patient variability resulting in unpredictable plasma concentrations. Some factors responsible for the variability such as age, body weight, concomitant medications, adherence and plasma protein concentrations change with time. While other factors like ethnicity, sex and genetics remain constant,[190] SNPs can alter the structure, expression and function of enzymes and transporters for which they code and may confer variability in the metabolism and distribution of darunavir.[191]

Darunavir is metabolized extensively by CYP3A4[181] and CYP3A5[192], whereas, ritonavir is metabolized mainly by CPY3A and by CYP2D6 to a lesser extent.[193] Darunavir is transported by OATP1B1, a member of solute carrier membrane transport proteins family, and is coded by *SLCO1B1* gene.[194] The transport of darunavir via OATP3A1 has not been confirmed by *in vitro* experimentation. However, using a pop-PK-model, involvement of OATP3A1 in the transport of darunavir has been suggested.[190]

PXR and CAR are xenobiotic sensors belonging to the nuclear receptor (NR) family and are coded by *NR1I2* and *NR1I3,* respectively. When activated, they control the expression of metabolic enzymes and transporters and help in detoxifying xenobiotics.[195] Darunavir plasma levels are influenced by *CYP3A4* and *ABCB1*, which are regulated by the PXR.[169] Similarly, CAR has been shown to up-regulate the *CYP3A4* gene and could potentially affect darunavir concentrations.[196]

Polymorphisms in the genes coding for metabolic enzymes and transporters could alter drug PK and cause serious adverse events or suboptimal efficacy, both resulting in treatment failure.[197] Hence, a thorough investigation of effects of SNPs in the genes that code for proteins that are involved in darunavir and ritonavir metabolism and

disposition is necessary.

In this study, candidate polymorphisms in *CYP3A4, CYP3A5, SLCO3A1, SLCO1B1, NR1I2* and *NR1I3* were assessed for their impact upon darunavir and ritonavir plasma levels. Data were collected from subjects enrolled in the NEAT001/ANRS143 study. The NEAT 001 / ANRS 143 is a Phase III, randomized, open labelled trial, conducted to analyse the efficacy of a novel NRTI-sparing dual combination of darunavir/r (ritonavir boosted) and raltegravir against a standard-of-care triple therapy of darunavir/r and TDF/emtricitabine in HIV-infected antiretroviral naïve subjects.[110]

2.2 Methods

2.2.1 Study Design and Samples Collection

Patients were a part of a multi-centre, open-label, randomized, two-year trial comparing two first-line regimens in HIV-infected antiretroviral naïve subjects: darunavir/r + TDF / emtricitabine vs. darunavir/r + raltegravir (ANRS 143/NEAT 001; ClinicalTrials.gov Identifier: NCT01066962). HIV-infected, treatment-naïve patients were recruited between August 2010 and September 2011 from 15 European countries (78 clinical sites). Individuals were eligible for enrolment if age ≥ 18 years, plasma HIV viral load was above 1000 copies/mL, CD4 count <500 cells/μL (except patients with symptomatic disease), HIV treatment naïve, and there was no previous or current evidence of major resistance mutations. Patients who were asymptomatic for any disease and had a CD4 count > 500 cells/μL were excluded from the study. Patients suffering from or requiring treatment for active OIs like TB and hepatitis, pregnant women, those with abnormal laboratory parameters or those with hepatic/renal impairment were not permitted to participate in the study. Ethical approval and written informed consent were obtained. A separate consent form for pharmacogenetics analysis was obtained. A total of 1.5 ml of full blood was stored in one ethylenediaminetetraacetic acid (EDTA) vial and labelled. The sample was then frozen and shipped at -80°C to a centralised laboratory for storage.

Patients were randomised (1:1) to receive ritonavir-boosted darunavir (800/100 mg once daily) with either TDF/emtricitabine (245/200 mg once daily) or raltegravir (400 mg twice daily; NRTI-sparing regimen).[110] Random, single blood samples were drawn at week 4 and 24 following therapy initiation and plasma was obtained for quantification of drug concentrations. Week 4 was chosen to assess the correlation

between drug plasma concentrations and adverse events if any. Week 24 was chosen to assess the correlation between drug plasma concentrations and treatment failure if any.

The blood samples collected for pharmacogenetics analysis were then shipped to the Department of Molecular and Clinical Pharmacology, University of Liverpool, United Kingdom (UK) for analysis. For the measurement of drug concentrations, 10 ml of blood was collected in EDTA tubes at week 4 and 24 and centrifuged to obtain plasma, which was stored at -80°C for 1 year until analysis. Thawing of the samples was kept to a minimum and done on ice to ensure DNA stability.

2.2.2 DNA Extraction and Genotyping

Total genomic DNA was extracted from patient blood using the QI Amp DNA mini kit (Qiagen, West Sussex, UK) according to manufacturer's instructions. The quality and quantity of DNA in the samples were spectrophotometrically using NanoDrop® (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The quality of DNA was tested by measuring the ratio of absorbance at 260 nm and 280 nm, with >1.8 to be accepted as pure. The DNA concentrations for all samples were above 20 ng/µl and stored at -20° C until analysis. Genotyping was performed by allelic discrimination real-time polymerase chain reaction (RT-PCR) assay on a DNA Engine Chromo4 system (Bio-Rad Laboratories, Hercules, CA) as previously described.[198] The PCR protocol involved an initial denaturation step at 95°C for 15 min, followed by 50 cycles of amplification at 95°C for 15 seconds and final annealing at 60°C for 1 min. The TaqMan® Genotyping Master Mix and assay numbers enumerated in **Table 2.1** were obtained from Life Technologies Ltd (Paisley, Renfrewshire, UK).

Table 2.1. TaqMan® assays

Gene and SNP	Reference SNP ID	Product ID
SLCO3A1 G>A	rs4294800	C__27008914_10
SLCO3A1 G>T	rs8027174	C__2901316_10
SLCO1B1 521 T>C	rs4149056	C__30633906_10
NR1I2 (PXR) 63396C>T	rs2472677	C__26079845_10
NR1I3 (CAR) 540G>A	rs2307424	C__25746794_20
CYP3A5 6986A>G	rs776746	C__26201809_30
CYP3A4 522-191C>T	rs35599367	C__26201809_30

The TaqMan assays contain pre-optimised PCR primers and two probes for allelic discrimination. The two TaqMan probes contains a FAM dye and a VIC dye label at the 5' end and minor groove binders and non-fluorescent quenchers at the 3' end. During a PCR, each probe anneals to its complementary sequence and is cleaved by the AmpliTaq Gold DNA polymerase. This separates the reporter dye from the quencher dye resulting in increased fluorescence of the reporter **(Figure 2.1)**.

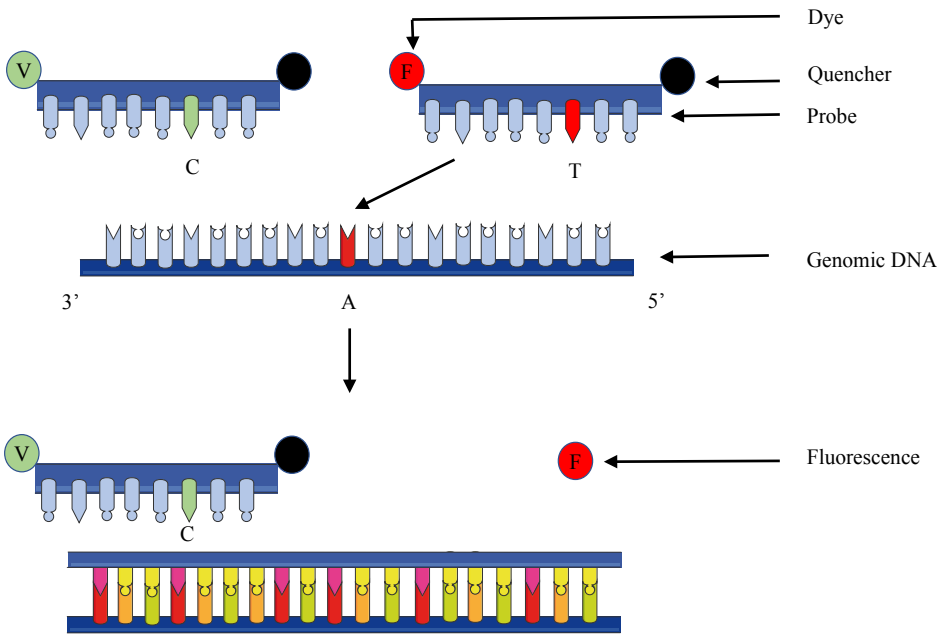

Figure 2.1 Real time PCR using TaqMan assays showing the annealing of probes to its complementary sequence resulting in reporter fluorescence.

Post-PCR, the allelic discrimination was analysed using a MiniOpticon System. In the amplification chart, a single threshold was manually assigned to all the samples as shown in **Figure 2.2** (Example of PCR done to assess the rs2307424). Based on the FAM and VIC endpoints, the samples were then grouped into wild, heterozygous or mutant homozygous (**Figure 2.3**).

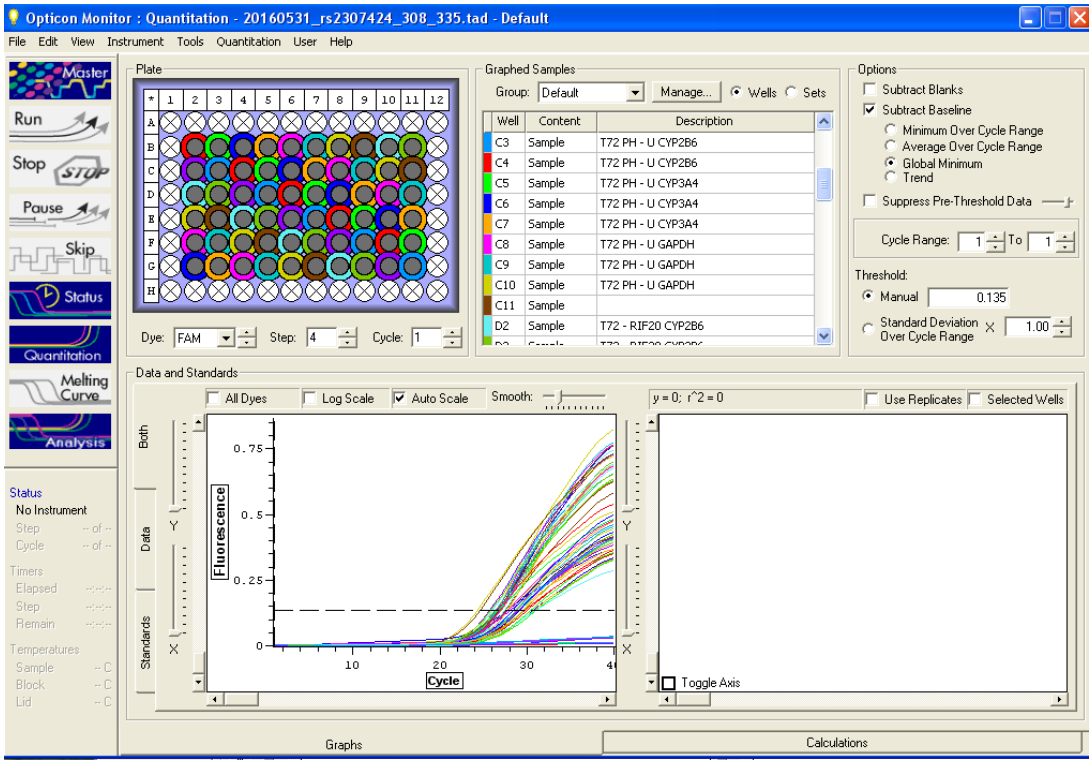

Figure 2.2. Analysis of realtime-PCR results for rs2307424. A single threshold is assigned to all the samples based on their amplification.

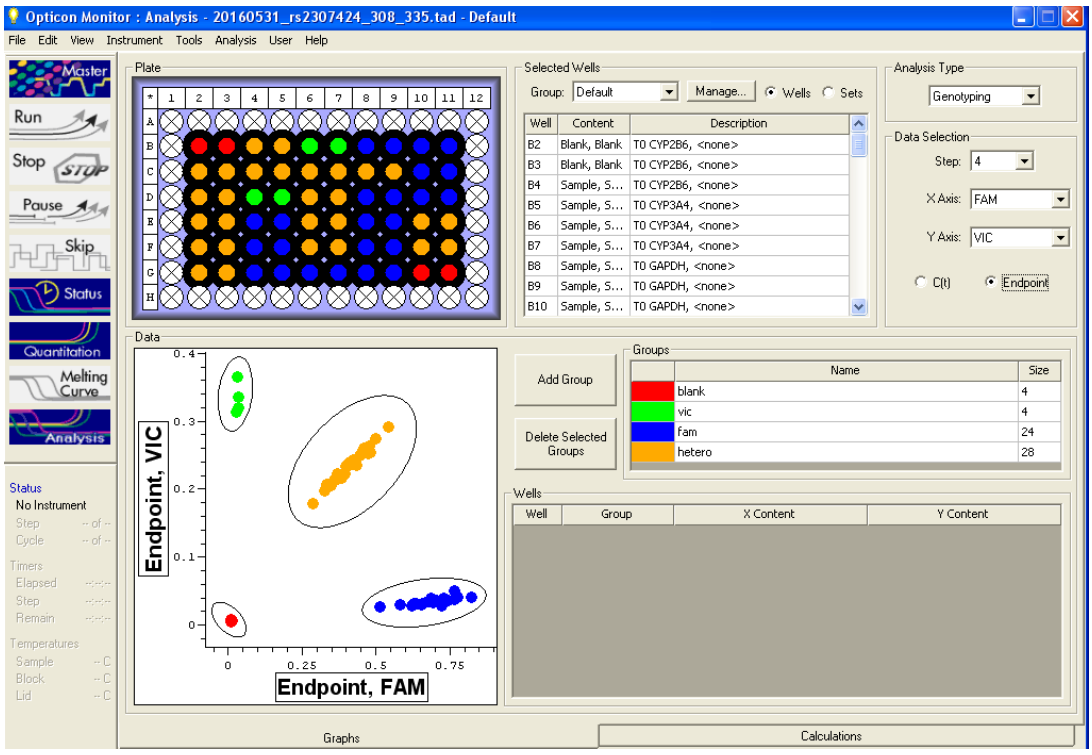

Figure 2.3 Grouping of samples into wild, heterozygous and mutant homozygous based on VIC and FAM endpoints.

2.2.3 Pharmacokinetic Analysis

Plasma drug concentrations were quantified at the Laboratory of Clinical Pharmacology and Pharmacogenetics, University of Turin (Turin, Italy) by validated HPLC-MS/MS methods. Lower limits of quantification (LLQ) were 0.0391 and 0.0098 mg/L for darunavir and ritonavir respectively.

2.2.4 Statistical Analysis

Hardy-Weinberg compliance was tested to confirm biological ascertainment using methods described by Rodriguez *et al*. This test was performed to examine the 'missingness' of subjects of a particular genotype, that can arise due to chance, genotyping errors or clinical biases.[199] The web program http://www.oege.org/software/hwe-mr-calc.shtml was used to carry out the tests. **Figure 2.4** is an example of Hardy Weinberg Equilibrium tested for *CYP3A4*22* (rs35599367) for darunavir at week 4 using the web program.

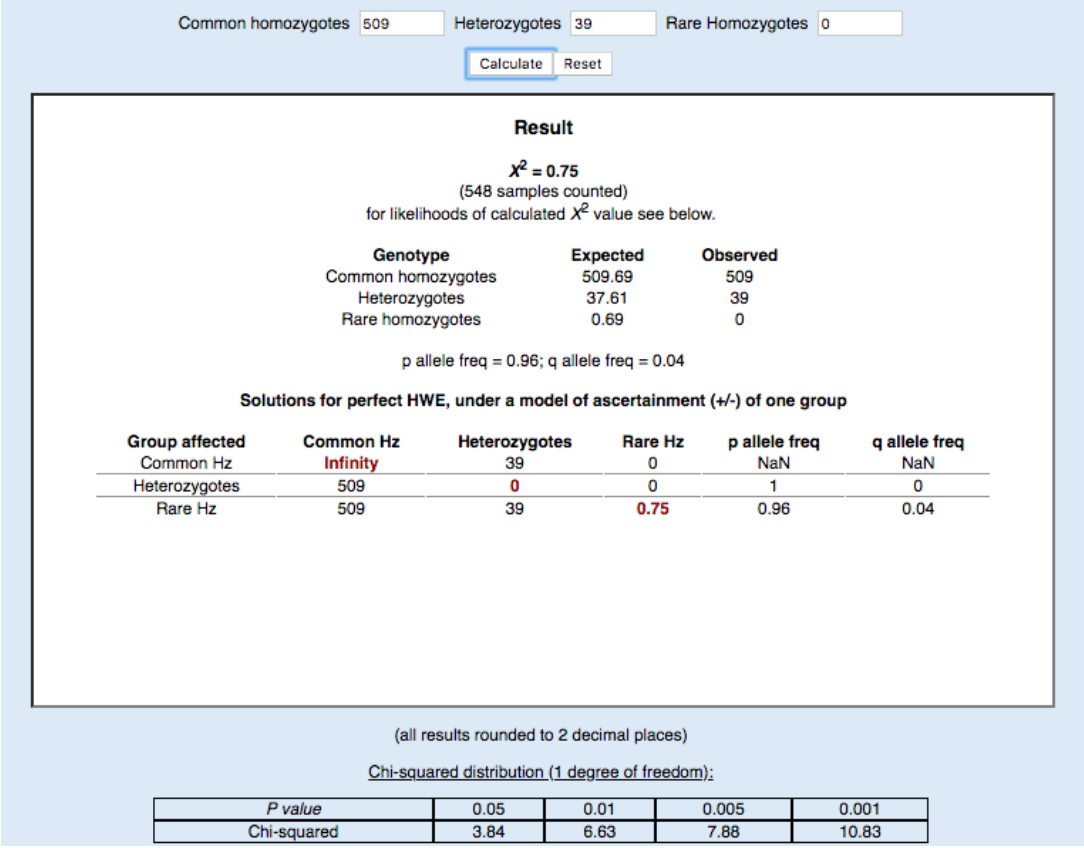

Figure 2.4. Hardy Weinberg Equilibrium test performed for *CYP3A4*22* (rs35599367) for darunavir at week 4. The x^2 0.75 has a P value > 0.05 indicating lack of difference between the observed frequency and population frequency.

Normality of the plasma concentrations of darunavir and ritonavir was tested using Kolmogorov-Smirnov test. Significance of SNPs on darunavir and ritonavir PK were performed separately for week 4 and week 24 sample collection. A univariate analysis was performed to identify the variables associated with darunavir and ritonavir plasma concentrations. The variables that had an association of P value (p) < 0.15 were included in the multivariate stepwise regression. A P value of < 0.05 was considered statistically significant in the multivariate analysis. Due to changes in drug concentrations with time, time post-dose was included in the multivariate analysis *a priori*. All statistical analysis was conducted using IBM SPSS Statistics v. 22.0.

2.3 Results

2.3.1 Patient Characteristics and Genotyping

Data were collected from 674 patients, of which 332 were randomised to the raltegravir arm and 342 were randomised to the TDF / emtricitabine arm of the study. Samples were excluded from darunavir and ritonavir analysis due to missing data for demographics, recorded time post-dose, genotype or drug plasma concentrations. Those with post-dose > 30 hours and drug concentrations below LLQ were also omitted from the analysis. Thus, a total of 548 for darunavir at week 4, 520 for darunavir at week 24, 554 for ritonavir at week 4 and 527 for ritonavir at week 24 patients were included in the analysis. All four groups used for analysis had an approximately equal number of patients in the raltegravir and TDF/emtricitabine arm, with a greater proportion of males to females. Patient demographics and clinical characteristics are described in **Table 2.2**. The allele frequencies for the tested SNPs are described in **Table 2.3**. All genotypes were in Hardy-Weinberg equilibrium except for *SLCO3A1* G>T (rs8027174) at week 4 and *CYP3A5*3* (rs776746) at week 4 and 24 for the analysis of darunavir and ritonavir.

Table 2.2. Clinical characteristics and demographics of patients included in the pharmacogenetic analysis of NEAT001/ANRS143 pharmacokinetic sub study stratified by study drug and week of sample collection (data expressed in median (range) unless stated otherwise)

Parameter	**Darunavir**		**Ritonavir**	
	Week 4	**Week 24**	**Week 4**	**Week 24**
Included for analysis (n)	548	520	554	527
Age (years)	37 (18-76)	37 (18-76)	37 (18-76)	37 (18-76)
Sex [n (%)]				
Male	484 (88.3)	464 (89.2)	489 (88.3)	470 (89.2)
Female	63 (11.5)	55 (10.6)	64 (11.6)	56 (10.6)
Transgender	1 (0.2)	1 (0.2)	1 (0.2)	1 (0.2)
Weight at randomisation (kg)	71.95 (41-135)	72 (44.3-135)	72 (41-135)	72 (44.3-135)
Height (cm)	176 (149-204)	176 (149-204)	176 (149-204)	176 (149-204)
Randomisation arm [n (%)]				
TDF/Emtricitabine	269 (49.1)	267 (51.3)	273 (49.3)	272 (51.6)
Raltegravir	279 (50.9)	253 (48.7)	281 (50.7)	255 (48.4)

Table 2.3. Allele frequencies for SNPs investigated for pharmacogenetic analysis; n (%)

SNP	Darunavir		Ritonavir	
	Week 4	Week 24	Week 4	Week 24
Number of patients	548	520	554	527
***CYP3A4*22* (rs35599367)**				
GG	509 (92.9)	480 (92.3)	514 (92.8)	487 (92.4)
GA	39 (7.1)	40 (7.7)	40 (7.2)	40 (7.6)
AA	0	0	0	0
***CYP3A5*3* (rs776746)**				
CC	402 (73.6)	378 (72.7)	408 (73.6)	383 (72.7)
CT	108 (19.7)	111 (21.3)	108 (19.5)	112 (21.3)
TT	38 (6.7)	31 (6)	38 (6.9)	32 (6)
***NR1I2* 63396C>T (rs2472677)**				
TT	178 (32.5)	172 (33.1)	180 (32.5)	173 (32.8)
CT	262 (47.8)	246 (47.3)	265 (47.8)	251 (47.6)
CC	108 (19.7)	102 (19.6)	109 (19.7)	103 (19.6)
***NR1I3* 540G>A (rs2307424)**				
GG	266 (48.5)	244 (46.9)	269 (48.6)	248 (47.1)
GA	224 (40.9)	223 (42.9)	227 (41)	224 (42.5)
AA	58 (10.6)	53 (10.2)	58 (10.4)	55 (10.5)
***SLCO1B1* 521T>C (rs4149056)**				
TT	393 (71.7)	374 (71.9)	399 (72)	378 (71.7)
CT	146 (26.6)	135 (26)	146 (8.3)	138 (26.2)
CC	9 (1.7)	11 (2.1)	9 (1.6)	11 (2.1)
***SLCO3A1* G>A (rs4294800)**				
GG	264 (48.2)	254 (48.8)	269 (48.6)	256 (48.6)
GA	229 (41.8)	212 (40.8)	229 (41.3)	216 (41)
AA	55 (10)	54 (10.4)	56 (10.1)	55 (10.4)
***SLCO3A1* G>T (rs8027174)**				
GG	460 (83.9)	442 (85)	466 (84.1)	447 (84.8)
GT	88 (16.1)	78 (15)	88 (15.9)	80 (15.2)
TT	0	0	0	0

2.3.2 Influence of SNPs on Darunavir Plasma Concentrations

According to the Kolmogorov-Smirnov test, the plasma concentrations of darunavir was not normally distributed. Hence, log concentrations of darunavir was used for analysis.

At week 4, a univariate analysis showed sex ($p = 0.096$), time of post-dose blood sample collection ($p < 0.0001$), ritonavir concentration ($p < 0.0001$), trial randomisation ($p = 0.013$), and *SLCO1B1* 521T>C ($p = 0.035$) had a significant effect on the darunavir plasma concentrations (**Table 2.4**). *CYP3A4*22* ($p = 0.859$), *CYP3A5*3* ($p = 0.814$), *NR1I2* 63396C>T ($p = 0.391$), *NR1I3* 540G>A ($p = 0.616$), *SLCO3A1* G>A ($p = 0.492$) and *SLCO3A1* G>T ($p = 0.225$) were not associated with darunavir plasma concentrations (**Figure 2.5**). On performing multivariate linear regression, time of the post-dose blood sample collection ($p < 0.0001$), ritonavir concentration ($p < 0.0001$), trial randomisation ($p = 0.008$) and *SLCO1B1* 521T>C ($p = 0.038$) showed statistically significant associations with darunavir plasma concentrations. Trial randomisation had a β value of 0.09 indicating darunavir plasma concentrations were significantly lower in patients receiving darunavir/r + raltegravir (**Figure 2.6**). Similarly, the β value for *SLCO1B1* 521T>C was 0.075; a significant higher concentration in patients containing the *SLCO1B1* 521T>C allele (**Figure 2.7).**

To further investigate the influence of trial randomisation on darunavir, the darunavir plasma concentrations collected between 20-30-hour post dose was analysed in patients receiving TDF/FTC against those receiving raltegravir (**Figure 2.8**). No significant differences ($p = 0.239$) were seen in darunavir concentration in the two groups.

Table 2.4. Association of patient characteristics and SNPs in *CYP3A4*, *CYP3A5*, *NR1I2*, *NR1I3*, *SLCO1B1* and *SLCO3A1* with log plasma darunavir concentrations in patients enrolled in NEAT001/ANRS143. Variables that were significant after univariate analysis (P < 0.15) were considered for multivariate regression analysis.

Variables	Week 4				Week 24			
	Univariate linear regression		Multivariate linear regression		Univariate linear regression		Multivariate linear regression	
	β	P value	β	P value	β	P value	β	P value
Age	0.018	0.63			-0.039	0.335		
Sex	-0.075	**0.096**	-0.016	0.663	0.038	0.398		
Weight at randomisation	-0.261	0.269			0.059	0.638		
Weight at blood collection	0.238	0.315			0.006	0.961		
Height	0.072	0.152			-0.063	0.216		
Time of post-dose blood collection	-0.237	**<0.0001**	-0.24	**<0.0001**	-0.303	**<0.0001**	-0.3	**<0.0001**
Ritonavir concentration	0.39	**<0.0001**	0.382	**<0.0001**	0.304	**<0.0001**	0.303	**<0.0001**
Trial Randomisation (RAL Vs TDF/FTC)	0.092	**0.013**	0.09	**0.008**	0.146	**<0.0001**	0.14	**<0.0001**
***CYP3A4*22* (rs35599367)**	0.007	0.859			0.001	0.985		
***CYP3A5*3* (rs776746)**	-0.009	0.814			-0.076	**0.053**	-0.06	0.126
***NR1I2* 63396C>T (rs2472677)**	0.031	0.391			-0.022	0.56		
***NR1I3* 540G>A (rs2307424)**	0.018	0.616			0.009	0.815		
***SLCO1B1* 521T>C (rs4149056)**	0.078	**0.035**	0.075	**0.038**	-0.032	0.401		
***SLCO3A1* G>A (rs4294800)**	-0.026	0.492			-0.014	0.721		
***SLCO3A1* G>T (rs8027174)**	-0.045	0.225			-0.018	0.634		

β is the regression coefficient and represents incremental change in log plasma darunavir concentration (conc.) per unit change in a patient characteristic

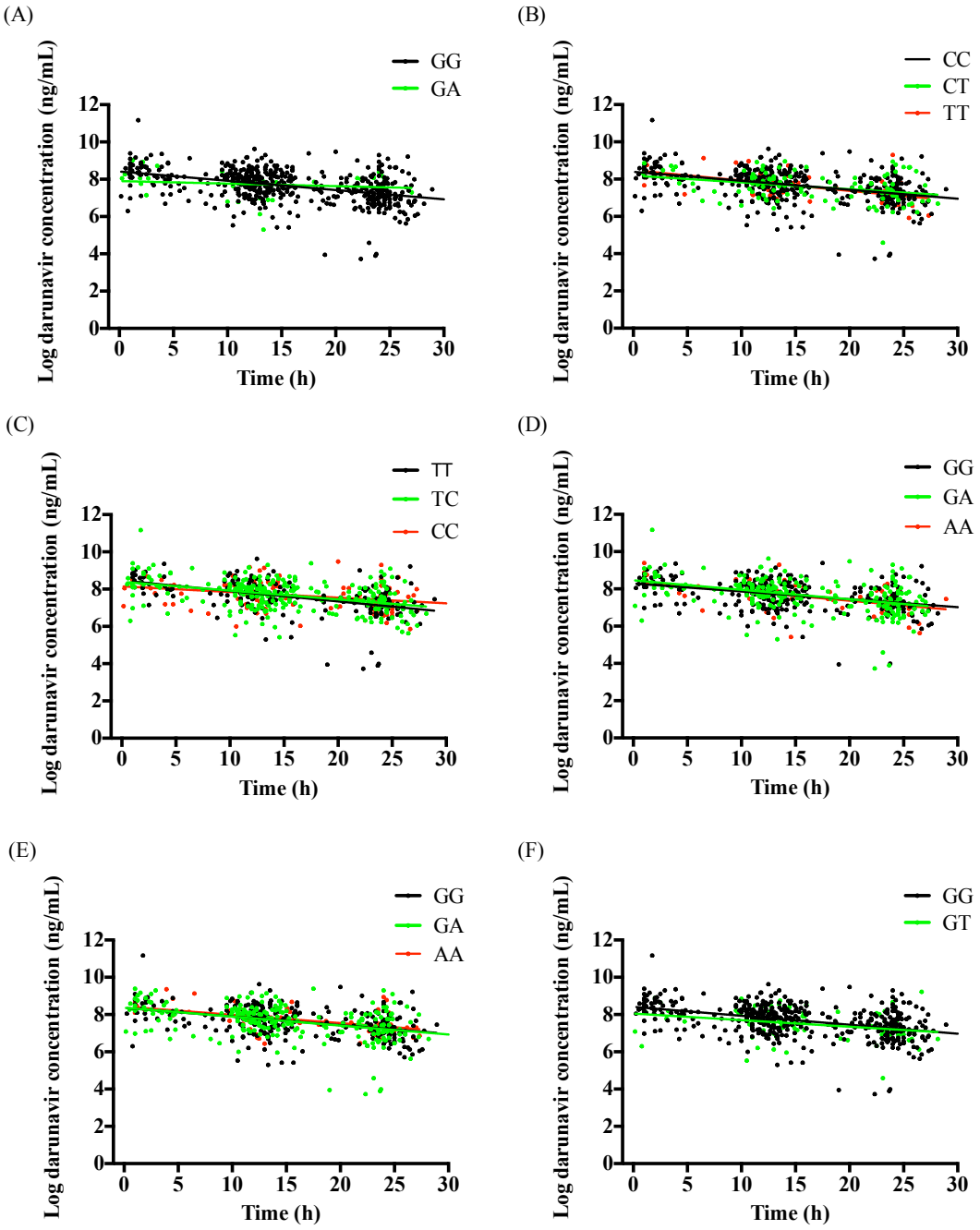

Figure 2.5. Influence of (A) *CYP3A4*22,* (B) *CYP3A5*3,* (C) *NR1I2* 63396C>T, (D) *NR1I3* 540G>A, (E) *SLCO3A1* G>A, (F) *SLCO3A1* G>T SNPs on darunavir $C_{0\text{-}24}$ concentrations at week 4. Solid lines represent mean darunavir plasma concentrations. Univariate regressions found no significant associations ($p < 0.15$) between the SNPs and darunavir $C_{0\text{-}24}$ concentrations.

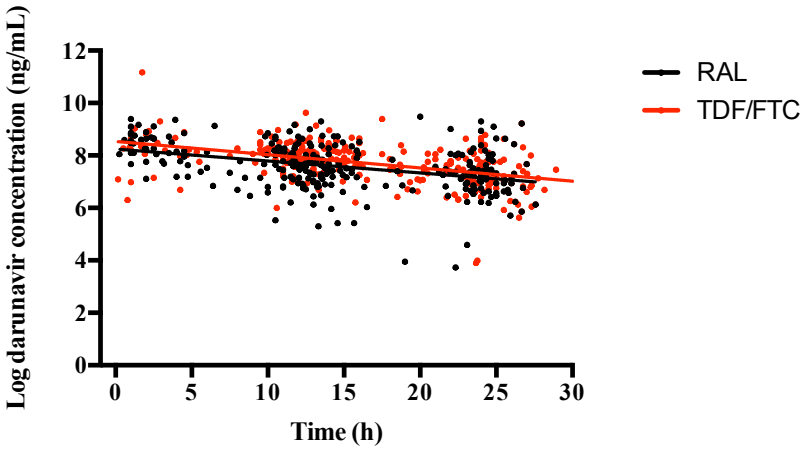

Figure 2.6. Influence of trial randomisation on $C_{0\text{-}24}$ darunavir concentrations at week 4. Solid lines represent mean darunavir plasma concentrations. Multivariate regression showed lower darunavir $C_{0\text{-}24}$ concentrations in patients receiving DRV/r + RAL ($p = 0.008$, $\beta = 0.09$).

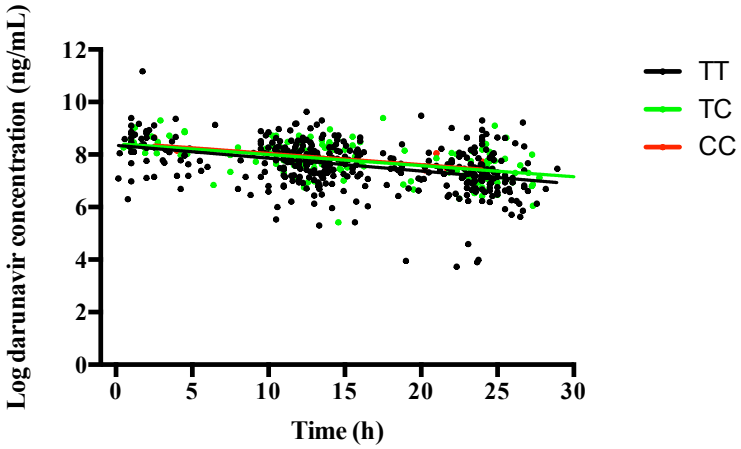

Figure 2.7. Influence of *SLCO1B1* 521T>C on darunavir $C_{0\text{-}24}$ concentrations at week 4. Solid lines represent mean darunavir plasma concentrations. Multivariate regression showed higher darunavir $C_{0\text{-}24}$ concentrations in patients with *SLCO1B1* 521T>C allele. ($p = 0.038$, $\beta = 0.075$).

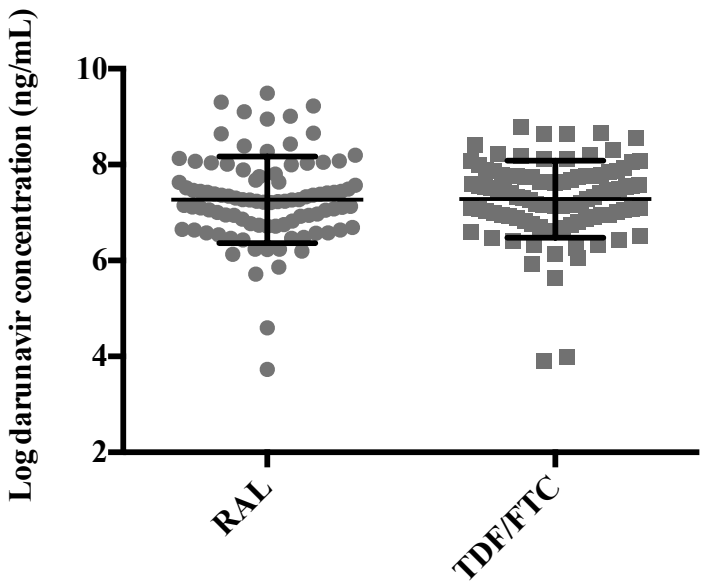

Figure 2.8. Influence of trial randomisation on darunavir plasma concentrations taken between 20-30-hour post-dose. Unpaired t-test showed no significant difference in the darunavir concentrations in the two groups. (p = 0.239).

At week 24, univariate analysis confirmed significant associations between darunavir plasma concentrations and time of post-dose blood sample collection ($p < 0.0001$), ritonavir concentration ($p < 0.0001$), trial randomisation ($p < 0.0001$) and *CYP3A5*3* ($p = 0.053$). *CYP3A4*22* ($p = 0.985$), *NR1I2* 63396C>T ($p = 0.56$), *NR1I3* 540G>A ($p = 0.815$), *SLCO1B1* 521T>C ($p = 0.401$), *SLCO3A1* G>A ($p = 0.721$) and *SLCO3A1* G>T ($p = 0.634$) did not affect the darunavir plasma concentrations (**Figure 2.9**). However, on performing multivariate regression, *CYP3A5*3* ($p = 0.126$) was no longer significantly associated with darunavir plasma concentrations (**Figure 2.10**). Whereas, post-dose blood sample collection ($p < 0.0001$), ritonavir concentration ($p < 0.0001$) and trial randomisation arm ($p < 0.0001$) were strongly associated with darunavir plasma concentrations. Trial randomisation had a β value of 0.14 indicating darunavir plasma concentrations were significantly lower in patients receiving darunavir/r + raltegravir (**Figure 2.11**).

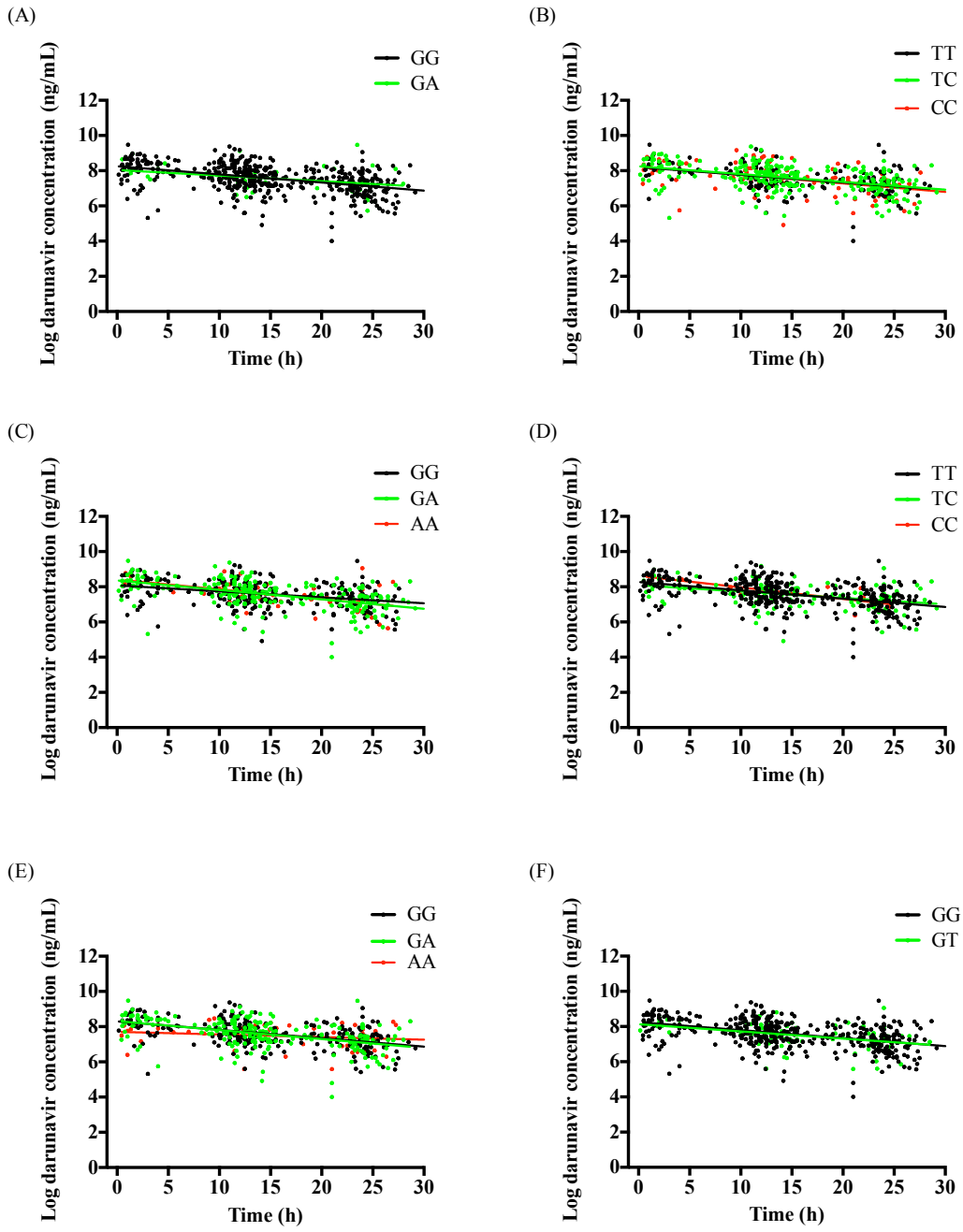

Figure 2.9. Influence of (A) *CYP3A4*22,* (B) *NR1I2* 63396C>T, (C) *NR1I3* 540G>A, (D) *SLCO1B1* 521T>C (E) *SLCO3A1* G>A, (F) *SLCO3A1* G>T on darunavir $C_{0\text{-}24}$ concentrations at week 24. Solid lines represent mean darunavir plasma concentrations. Univariate regressions found no significant associations ($p < 0.15$) between the SNPs and darunavir $C_{0\text{-}24}$ concentrations.

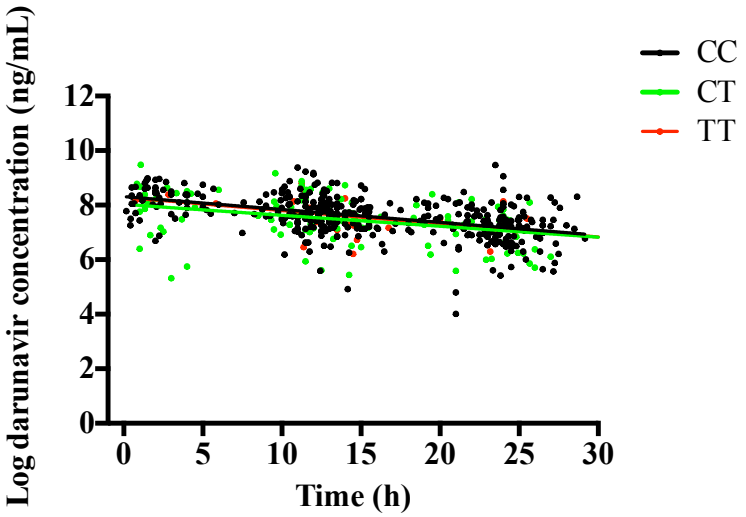

Figure 2.10. Influence of *CYP3A5*3* on darunavir $C_{0\text{-}24}$ concentrations at week 24. Solid lines represent mean darunavir plasma concentrations. Multivariate regression found no associations (p = 0.126) between *CYP3A5*3* and darunavir $C_{0\text{-}24}$ concentrations

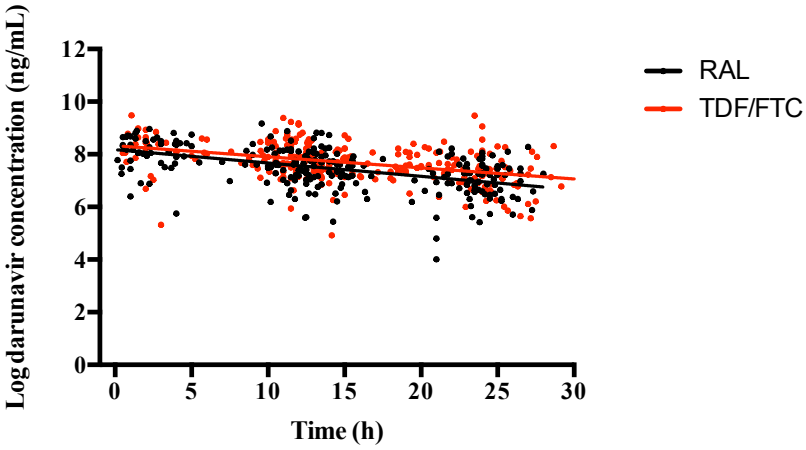

Figure 2.11. Influence of trial randomisation on darunavir $C_{0\text{-}24}$ concentrations at week 24. Solid lines represent mean darunavir plasma concentrations. Multivariate regression showed lower darunavir $C_{0\text{-}24}$ in patients receiving DRV/r + RAL ($p < 0.0001$, $\beta = 0.14$)

2.3.3 Influence of SNPs on Ritonavir Plasma Concentrations

According to the Kolmogorov-Smirnov test, the plasma concentrations of ritonavir was not normally distributed. Hence, log concentrations of ritonavir were used for analysis.

At week 4, a univariate regression showed height ($p = 0.006$), time of post-dose blood collection ($p < 0.0001$), trial randomisation ($p = 0.088$) and *SLCO3A1* G>A ($p = 0.077$) had a significant effect ($p < 0.15$) on the ritonavir plasma concentrations (**Table 2.5**). *CYP3A4*22* ($p = 0.458$), *CYP3A5*3* ($p = 0.566$), *NR1I2* 63396C>T ($p = 0.665$), *NR1I3* 540G>A ($p = 0.815$), *SLCO1B1* 521T>C ($p = 0.803$) and *SLCO3A1* G>T ($p = 0.299$) did not significantly influence ritonavir plasma concentrations (**Figure 2.12**). On performing multivariate regression, the influence of height ($p = 0.003$), time of post-dose blood collection ($p < 0.0001$) and *SLCO3A1* G>A ($p = 0.044$) on ritonavir plasma concentrations remained significant. *SLCO3A1* G>A had a β value of 0.139, indicating higher concentrations of ritonavir in patients carrying the *SLCO3A1* G>A allele (**Figure 2.13**).

Table 2.5. Association of patient characteristics and SNPs in *CYP3A4*, *CYP3A5*, *NR1I2*, *NR1I3*, *SLCO1B1* and *SLCO3A1* with log plasma ritonavir concentrations in patients enrolled in NEAT001/ANRS143. Variables that were significant after univariate analysis (P < 0.15) were considered for multivariate regression analysis.

Patient Characteristics	**Week 4**				**Week 24**			
	Univariate linear regression		**Multivariate linear regression**		**Univariate linear regression**		**Multivariate linear regression**	
	β	**P value**	**β**	**P value**	**β**	**P value**	**β**	**P value**
Age	-0.049	0.22	-	-	-0.047	0.248	-	-
Sex	0.019	0.682	-	-	0.016	0.723	-	-
Weight at randomisation	0.207	0.399	-	-	0.301	**0.018**	-0.001	0.975
Weight at blood collection	-0.178	0.47	-	-	-0.247	**0.052**	-0.029	0.452
Height	-0.143	**0.006**	-0.112	**0.003**	-0.116	**0.023**	-0.008	0.076
Time of post-dose blood collection	-0.459	**<0.0001**	-0.453	**<0.0001**	-0.497	**<0.0001**	-0.492	**<0.0001**
Trial Randomisation (RAL Vs TDF/FTC)	0.066	**0.088**	0.066	0.081	0.124	**0.001**	0.13	**<0.0001**
***CYP3A4*22* (rs35599367)**	-0.029	0.458	-	-	-0.024	0.528	-	-
***CYP3A5*3* (rs776746)**	-0.023	0.566	-	-	0.012	0.757	-	-
***NR1I2* 63396C>T (rs2472677)**	-0.017	0.665	-	-	0.061	**0.111**	0.071	0.062
***NR1I3* 540G>A (rs2307424)**	-0.027	0.472	-	-	0.011	0.765	-	-
***SLCO1B1* 521T>C (rs4149056)**	0.01	0.803	-	-	0.081	**0.039**	0.072	0.058
***SLCO3A1* G>A (rs4294800)**	0.069	**0.077**	0.139	**0.044**	-0.02	0.608	-	-
***SLCO3A1* G>T (rs8027174)**	-0.041	0.299	-	-	-0.013	0.736	-	-

β is the regression coefficient and represents incremental change in log plasma ritonavir concentration (conc.) per unit change in a patient characteristics

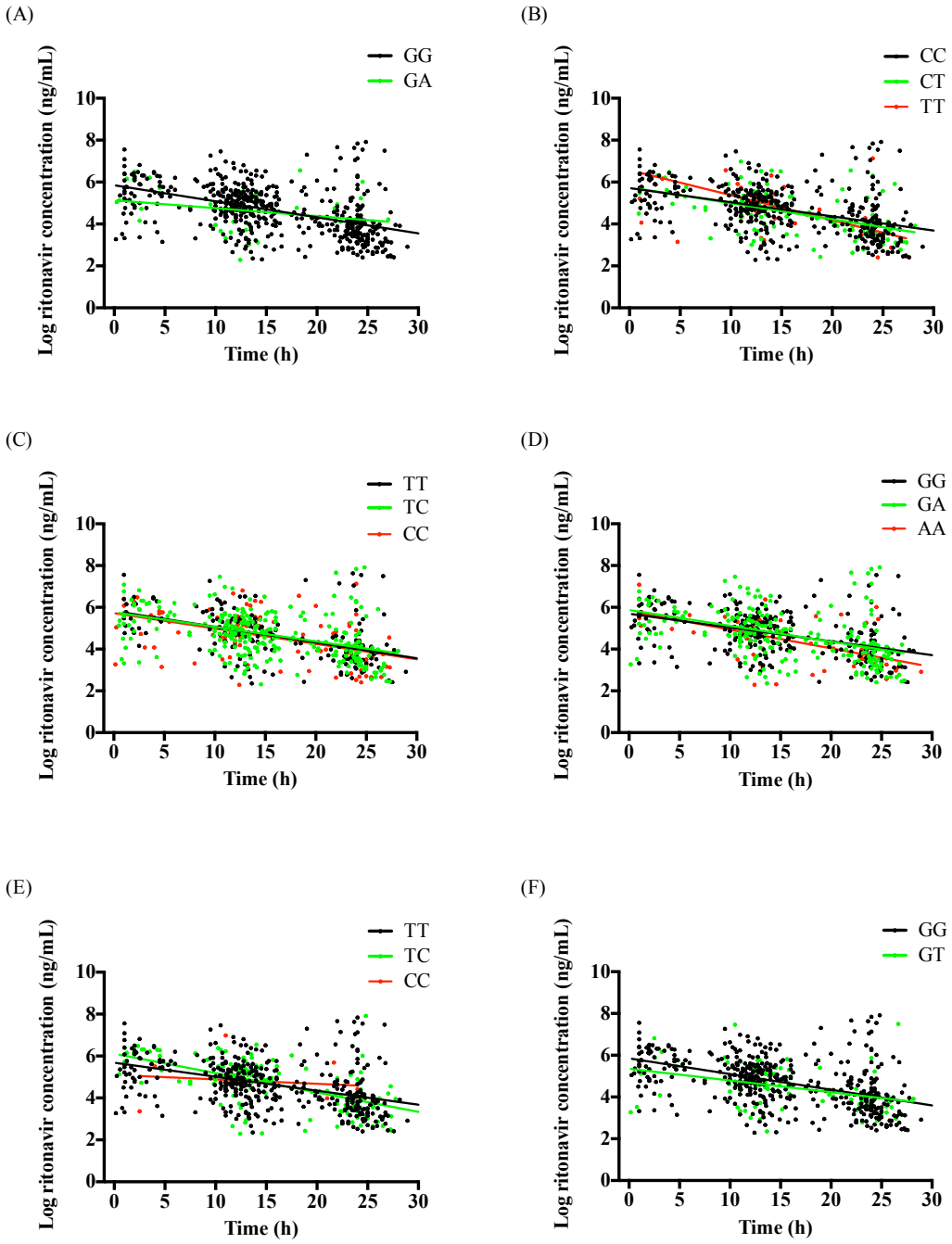

Figure 2.12. Influence of (A) *CYP3A4*22,* (B) *CYP3A5*3,* (C) *NR1I2* 63396C>T, (D) *NR1I3* 540G>A, (E) *SLCO1B1* 521T>C, (F) *SLCO3A1* G>T on ritonavir $C_{0\text{-}24}$ concentrations at week 4. Solid lines represent mean ritonavir plasma concentrations. Univariate regression found no significant associations ($p < 0.15$) between the SNPs and ritonavir $C_{0\text{-}24}$ concentration.

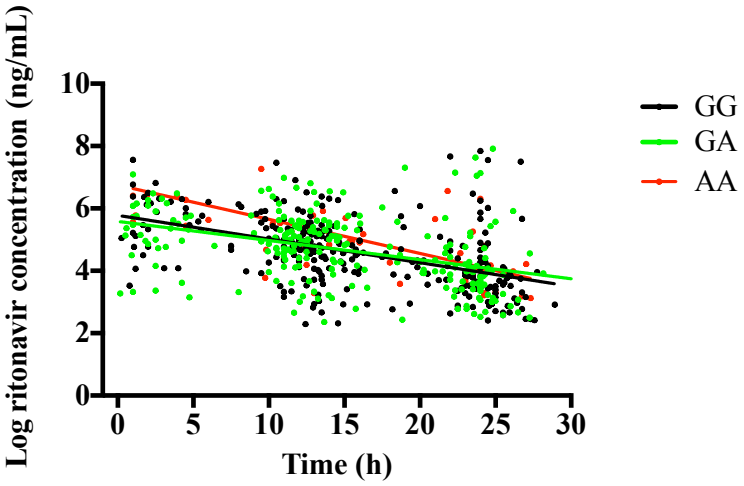

Figure 2.13. Influence of *SLCO3A1* G>A on $C_{0\text{-}24}$ ritonavir concentrations at week 4. Solid lines represent mean ritonavir plasma concentrations. Multivariate regression showed higher ritonavir $C_{0\text{-}24}$ in patients with *SLCO3A1* G>A. ($p = 0.044$, $\beta = 0.139$).

On performing a univariate regression at week 24, weight at randomisation ($p = 0.018$), weight at the time of blood collection ($p = 0.052$), height ($p = 0.023$), time of post-dose blood collection ($p < 0.0001$), trial randomisation ($p = 0.001$), *NR1I2* 63396C>T ($p = 0.111$) and *SLCO1B1* 521T>C ($p = 0.039$) were associated with ritonavir plasma concentrations. *CYP3A4*22* ($p = 0.528$), *CYP3A5*3* ($p = 0.757$), *NR1I3* 540G>A ($p = 0.765$), *SLCO3A1* G>A ($p = 0.608$) and *SLCO3A1* G>T ($p = 0.736$) did not influence ritonavir plasma concentrations (**Figure 2.14**). On performing a multivariate regression, time of post-dose blood sample collection ($p < 0.0001$) and trial randomisation ($p < 0.0001$) were the only covariates to be significantly associated with ritonavir concentrations. *NR1I2* 63396C>T ($p = 0.062$, **Figure 2.15 (A)**) and *SLCO1B1* 521T>C ($p = 0.058$, **Figure 2.15 (B)**) were not associated with ritonavir plasma concentrations. The β value of 0.13 indicates a significant lower ritonavir concentration in patients receiving darunavir/r + raltegravir (**Figure 2.16**).

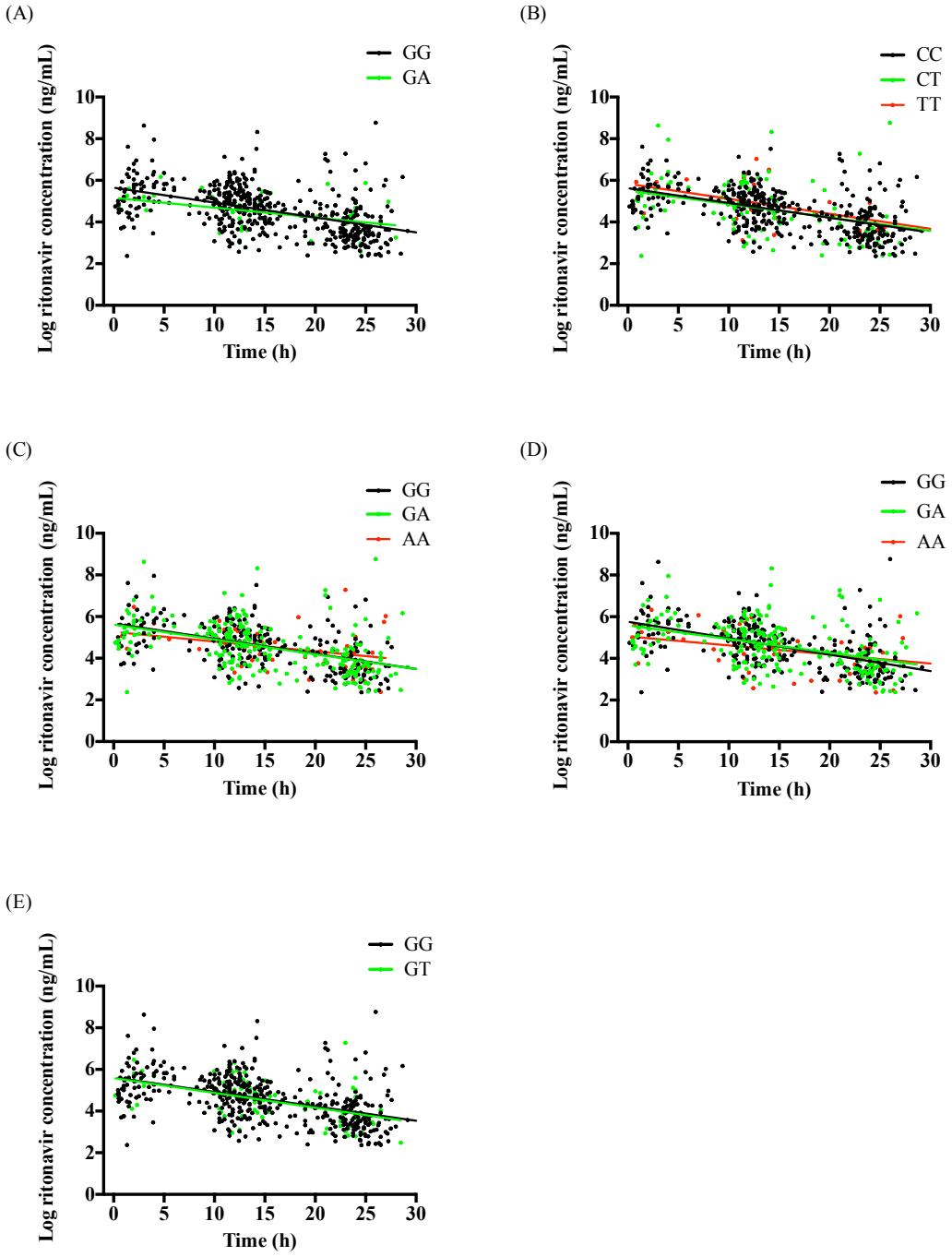

Figure 2.14. Influence of $C_{0\text{-}24}$ (A) *CYP3A4*22,* (B) *CYP3A5*3,* (C) *NR1I3* 540G>A, (D) *SLCO3A1* G>A and (E) *SLCO3A1* G>T on ritonavir $C_{0\text{-}24}$ concentrations at week 24. Solid lines represent mean ritonavir plasma concentrations. Univariate regression found no significant associations between the SNPs and ritonavir $C_{0\text{-}24}$ concentrations

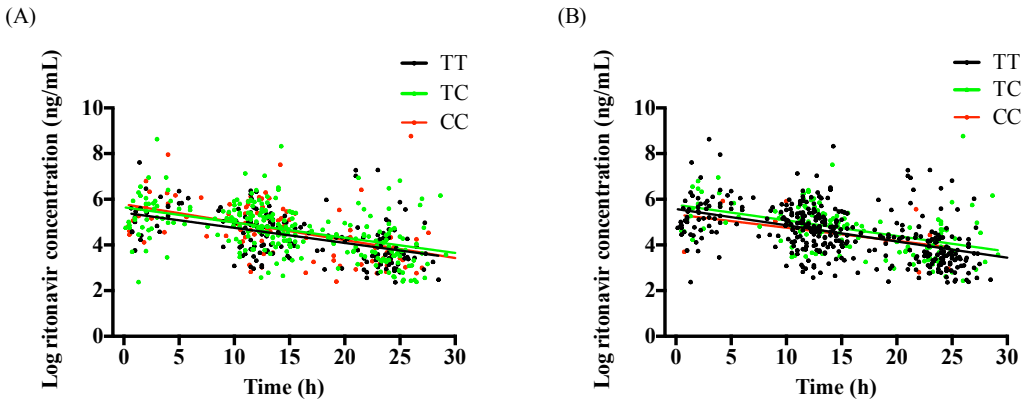

Figure 2.15. Influence of (A) *NR1I2* 63396C>T and (B) *SLCO1B1* 521T>C on $C_{0\text{-}24}$ ritonavir concentrations at week 24. Solid lines represent mean ritonavir plasma concentrations. Multivariate regression did not show any significant associations between *NR1I2* 63396C>T ($p = 0.062$) and *SLCO1B1* 521T>C ($p = 0.058$) with $C_{0\text{-}24}$ ritonavir concentrations.

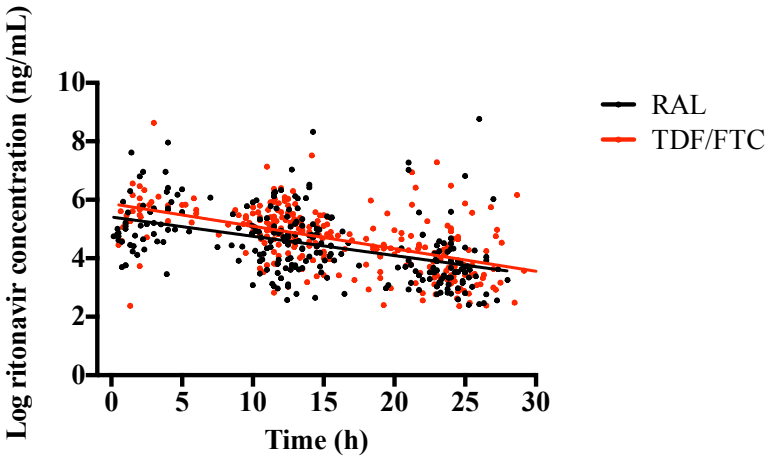

Figure 2.16. Influence of trial randomisation on $C_{0\text{-}24}$ ritonavir concentrations at week 24. Solid lines represent mean ritonavir plasma concentrations. Multivariate regression showed lower ritonavir $C_{0\text{-}24}$ in patients receiving DRV/r + RAL ($p < 0.0001$, $\beta = 0.13$).

2.4 Discussion

All genotypes were in Hardy-Weinberg equilibrium except for *SLCO3A1* G>T (rs8027174) at week 4 and *CYP3A5*3* (rs776746) at week 4 and 24 for the analysis of darunavir and ritonavir. This indicates missingness of genotypes due to chance, genotyping assay errors or clinical biases. The frequency of polymorphisms for the two genotypes were not representative of the general population. Hence, the results from these genotypes should be interpreted with caution.

This study is the first to investigate the effects of clinically relevant SNPs on darunavir PK in a large population. The plasma concentration of darunavir at week 4 (**Figure. 2.6**) and week 24 (**Figure 2.11**), and the ritonavir plasma concentration at week 24 (**Figure 2.16**) was significantly lower in patients receiving darunavir/r + raltegravir compared to those receiving darunavir/r + TDF/emtricitabine. This is suggestive of a DDI due to co-administration of either raltegravir or TDF or both. Hoetelmans *et al*. demonstrated that although clinically insignificant, there was an increase in the C_{max}, AUC and C_{min} of darunavir by 16%, 21% and 24% in the presence of TDF.[200]

The PK of darunavir and raltegravir in terms of known induction/inhibition of metabolic enzymes and transporters are exclusive to each other, so a DDI is not expected. However, a considerable number of studies have demonstrated a fall in darunavir concentrations in the presence of raltegravir.[201] Cattaneo *et al*. have shown a 40% reduction in C_{max} and a 60% increase in clearance of darunavir.[202] Additionally, raltegravir has also been shown to decrease the AUC and C_{min} of other PIs such as atazanavir.[203] The results from the NEAT001/ANRS143 study showed a higher percentage of treatment failures in patients receiving raltegravir compared to those receiving TDF/emtricitabine, which was correlated to below detectable levels of

darunavir.[110] Moreover, a significantly large number of viral mutations were seen in patients receiving raltegravir compared to no mutations in patients receiving TDF/emtrivitabine.[204]

DDIs causing a change in ritonavir concentration can impact the PI it boosts. For example, increased clearance of ritonavir could lead to loss of pharmaco-enhancement of PIs resulting in higher rates of metabolism and elimination.[205] Since ritonavir is a PI and has pharmacokinetic properties similar to darunavir and atazanavir, it is possible that low ritonavir concentrations in patients receiving raltegravir are a result of the same DDIs seen with other PIs. These findings may warrant re-examination of the pharmacokinetic profile of all PIs as well as raltegravir.

*CYP3A4*22* (522-191 C>T; rs35599367) is associated with low expression and activity of CYP3A4.[206] It was found to be responsible for a 31.7 – 33.6 % reduction in midazolam apparent clearance when compared to *CYP3A4*1*.[207] Similarly, a 2.3-fold higher lopinavir trough concentration was seen in patients homozygous for *CYP3A4*22* compared to non-carriers.[208] The study was conducted in 375 patients and the analysis was done using a population pharmacokinetic approach. Our findings, using multivariate regression, indicate that *CYP3A4*22* did not have a significant effect on darunavir and ritonavir plasma levels.

The *CYP3A5*3* (6986 A>G; rs776746) allele is associated with loss of function of CYP3A5 and has been shown to cause a 79% decrease in maraviroc metabolite formation compared to wild type *CYP3A5*1*.[209] Similarly, *CYP3A5*3* was associated with increased tacrolimus concentration in the Chinese population.[210] Our study did not establish any significance between *CYP3A5*3* and darunavir and ritonavir plasma concentrations.

The *SLCO1B1* 521T>C (rs4149056) SNP is found in high frequency in Caucasians and was demonstrated to be associated with higher lopinavir concentrations in patients treated for HIV.[194] Similarly, in our study, patients carrying the *SLCO1B1* 521T>C had significantly higher ($p = 0.038$, $\beta = 0.075$) concentration of darunavir (**Figure. 2.7**). However, this effect was not observed at week 24. The steady-state of darunavir is reached within three days[211] and would not be responsible for PK differences at week 4 and 24. Alterations in the activity and expression of enzymes, transporters and plasma proteins of HIV-infected individuals has been previously reported.[212,213] With the fall in viral load and rebuilding of immunity due to ART, these parameters responsible for pharmacokinetics are restored. This could explain the disparity in the influence of *SLCO1B1* 521T>C on darunavir plasma levels at week 4 and week 24. Further investigations are required to investigate this for better understanding of the role of SNPs at various stages of HIV treatment.

The *NR1I2 (PXR)* 63396C>T (rs2472677) allele increases *NR1I2* expression and is associated with a suboptimal atazanavir C_{trough} and 17.2% increase in atazanavir clearance. This study was conducted using 323 samples form 182 randomly selected patients. The analysis was done using population pharmacokinetic method.[169] Similarly, SNPs in *NR1I3* have been associated with significant alteration in drug concentrations. For example, it was seen that *NR1I3* (*CAR*) 540G>A (rs2307424) was associated with low efavirenz plasma concentrations[214] and in some cases treatment discontinuation.[170] The effects of these polymorphisms, however, were not seen in our analysis.

To our knowledge, the transport of darunavir via OATP3A1 has not been demonstrated by *in vitro* experimentation. A recent Physiologically based pharmacokinetic (PBPK) model that investigated darunavir PK in pregnant women

has suggested involvement of hepatic transporters in the disposition of darunavir. Molto *et al*. using a pop-PK-model, showed a significant effect of *SLCO3A1* G > A (rs4294800) and *SLCO3A1* G > T (rs8027174) polymorphisms, that code for OATP3A1, on darunavir clearance and apparent volume of distribution.[190] In our study, *SLCO3A1* G > A (rs4294800) was associated with higher ritonavir plasma concentrations at week 4 (**Figure 2.13**). These findings necessitate an investigation of the role of OATP3A1 in darunavir PK.

The plasma concentrations of darunavir and ritonavir are inversely proportional to the time elapsed since previous dose when blood was collected for measurement of drug plasma concentration. Hence, time of post-dose collection was significant for all the drug plasma concentrations. Darunavir is boosted by ritonavir and the boosting is directly proportional to the ritonavir concentrations. Moreover, as time elapses, the plasma concentrations of ritonavir and darunavir reduce. Therefore, ritonavir concentrations were significant for darunavir plasma concentrations.

A limitation of this study is that a multivariate linear regression is not the ideal statistical method to analyse drug concentrations which have variable post-dose collection times. A nonlinear mixed effect modelling (NONMEM) estimation would be more appropriate for analysing the effects of the tested polymorphisms on pharmacokinetic parameters such as clearance, AUC, C_{max} and C_{trough}. These efforts are currently under way with the collaboration of clinicians, statisticians and pharmacologists involved in the trial. These results should be interpreted with precautions since the influence of polymorphisms and trial randomisation was significant on plasma concentrations and may not have biological implications. Additional investigations are needed to assess the influence of genetic polymorphisms on the pharmacodynamic properties of the tested drugs.

Our study was the largest, multi-centric study till date to assess the influence of genetic polymorphisms on the drugs administered in the study. Any polymorphism that showed a statistically significant influence, irrespective of the sample size and reproducibility of the results. Moreover, our study assessed the influence on plasma concentrations taken at a single time point at week 4 and 24. These differences in the number of clinical sites, sample size and methods for measuring drug plasma concentrations may be responsible for the discrepancies in the results observed in literature and our results. More studies with robust methodologies such as therapeutic drug monitoring and next generation sequencing are needed to establish the influence of the polymorphisms on the drug plasma concentration.

In conclusion, a significant reduction in the darunavir and ritonavir concentrations was seen when administered with raltegravir. Moreover, it is likely that darunavir is a substrate of OATP3A1. A re-investigation of pharmacokinetic properties of darunavir, ritonavir, TDF and raltegravir to explain the DDIs is needed. Although some of the tested polymorphisms were found to have a significant impact at week 4, the influence was not seen at week 24. The clinical significance of the tested polymorphisms therefore should be interpreted with caution.

Chapter 3

Pharmacogenetics of Tenofovir, Emtricitabine and Raltegravir in HIV-infected Adults: a sub-study of NEAT001/ANRS143

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3.4 Discussion

3.1 Introduction

In the early years of ART, zidovudine and lamivudine were administered by monotherapy to combat HIV.[215,216] It was soon discovered that a combination of two NRTIs were better at inhibiting HIV replication, increasing CD4 cell counts and reducing AIDS-related illnesses.[52,51] Multiple trials demonstrated safety and efficacy of zidovudine combined with lamivudine, after which this combination was considered the backbone for ART for many years.[217] However, this combination was subsequently replaced by a new backbone comprising of TDF and emtricitabine. TDF/emtricitabine as a backbone shows superior viral load suppression and is better tolerated than zidovudine/lamivudine.[218,219] In 2004, the FDA approved the combination of these two drugs as a fixed-dose combination (Truvada®) for once daily dosing.[220] Recently, Truvada® was also approved by the FDA for the use of PrEP.[221]

Many NRTIs exhibit issues with safety resulting in low tolerability and treatment discontinuation. To obviate these complications, NRTI-sparing regimens are being explored for the treatment of HIV.[188,189] A Phase III, randomized, open labelled trial called NEAT 001 / ANRS 143, was conducted to analyse the efficacy of a novel NRTI-sparing dual combination of darunavir/r and raltegravir against a standard-of-care triple therapy of darunavir/r and TDF/emtricitabine in HIV-infected antiretroviral naïve subjects.[110] In this chapter, the data collected from patients in this trial were used to explore the pharmacokinetic properties of tenofovir, emtricitabine and raltegravir.

TDF, an ester prodrug of NRTI tenofovir, was synthesized to overcome the low absorption and bioavailability of tenofovir.[222] TDF is hydrolysed to tenofovir

intracellularly and phosphorylated to its active metabolite tenofovir diphosphate.[223] There is no involvement of CYP enzymes in the metabolism of tenofovir, hence there are no expected DDIs with drugs whose metabolism is CYP-mediated.[224] Tenofovir is excreted unchanged by the kidneys and this gives rise to renal tubular toxicity and renal impairement.[225] The exact mechanism of toxicity is unknown. Hall *et al*. have suggested a mitochondrial toxicity exhibited by tenofovir directly, interference with tubular function or both could be the reasons for the observed toxicity.[226] Tenofovir is transported in the proximal tubules by the ABCC4 (MRP4),[227] ABCC11 (MRP8),[228] ABCC10 (MRP7),[229] OAT1 and OAT3.[230] Additionally, polymorphisms found in *ABCC10* were shown to influence the PK of tenofovir.[231] DDIs between tenofovir and other ARVs such as ritonavir,[232] lopinavir,[233] nevirapine and efavirenz[234] have been reported. These interactions are also shown to happen at the intra-cellular level involving influx transporter on the CD4+ cells. For example, Liptrott *et al.* demonstrated a 39% and 73% reduction in intra-cellular nevirapine in the presence of tenofovir in CD4+ cells and monocyte-derived macrophages. Similarly, nevirapine caused a 57% decrease in tenofovir accumulation.[123]

Emtricitabine is a fluorinated derivative of lamivudine and gets incorporated into HIV-1 DNA, resulting in chain termination.[235] Like tenofovir, there is no involvement of CYP enzymes in the metabolism of emtricitabine and it is excreted mostly unchanged.[236] Emtricitabine is a substrate of the MATE1 transporter by which it is excreted with no involvement of other transporters such as OCT1, OCT2, MRP2 that are found in renal tubules.[237] Toxicities and DDIs with emtricitabine are rare.[238,134]

Raltegravir is an integrase inhibitor that inhibits the insertion of HIV-1 complementary DNA into the host genome. Due to the difference in the mechanism of action of raltegravir, it shows good efficacy against multidrug-resistant HIV.[239] It

is metabolized by the UGT1A1 by glucuronidation with no involvement of CYP enzymes.[240] Raltegravir is a substrate of OAT1,[241] but evidence of involvement of P-gp and BCRP in its transport is inconsistent.[242]

The efficacy of these drugs is to variable degrees dependent on the metabolic enzymes and transporters and a change in their structure or function may affect the metabolism and distribution of the drugs.[191] Polymorphisms in the genes coding for the enzymes and transporters can lead to adverse events or suboptimal efficacy, both resulting in treatment failure.[197] Hence, a thorough investigation of effects of SNPs in the genes is necessary.

In this study, the effects of SNPs found in *ABCC2*, *ABCC10*, *SLC47A1*, *SLC22A6* and *UGT1A1*, that are been shown to have a clinically significant impact in other areas, on the plasma concentrations of tenofovir, emtricitabine and raltegravir were assessed.

3.2 Methods

3.2.1 Study Design and Samples Collection

The study design and the sample collection for the NEAT001/ANRS143 has been described in detail in Chapter 2, Section 2.2.1.

3.2.2 DNA Extraction and Genotyping

DNA extraction and genotyping were carried out using methods that have been described in Chapter 2, Section 2.2.2. The TaqMan® Genotyping assays used for analysis of SNPs are enumerated in **Table 3.1**

UGT1A1 polymorphisms were genotyped using the Sequenom MassARRAY platform and iPLEX Pro UGT1A1-TA assays (Sequenom Laboratories, San Diego, CA, USA) according to manufacturer practices. Similar to previous methods, 20 ng of genomic DNA was amplified by PCR and then treated with shrimp alkaline phosphatase to inactivate unincorporated nucleotides. Using iPLEX Gold Reaction Cocktail, single base extension reaction was performed followed by spotting onto SpectroCHIP II.[243] Data were analysed by MassARRAY TYPER software (v. 4.0.20, Sequenom Laboratories).

Table 3.1 TaqMan® Assays

Gene and SNP	Reference SNP ID	Product ID
ABCC2 24C>T	rs717620	C__2814642_10
ABCC2 1249 G>A	rs2273697	C__22272980_20
ABCC10 526G>A	rs9349256	C__1701942_10
ABCC10 2843 T>C	rs2125739	C__16173668_10
SLC47A1 G>A	rs2289669	C__15882280_10
SLC22A6 453 G>A	rs4149170	Custom Designed
SLC22A6 728 C>T	rs11568626	C__2558602_40

3.2.3 Pharmacokinetic Analysis

Plasma drug concentrations were quantified at the Laboratory of Clinical Pharmacology and Pharmacogenetics, University of Turin (Turin, Italy) by fully validated HPLC-MS/MS methods. Lower limits of quantification (LLQ) were 5.6 μg/mL for tenofovir and 1.17 μg/mL for emtricitabine and raltegravir.

3.2.4 Statistical Analysis

Hardy-Weinberg compliance was tested using methods described by Rodriguez *et al*.[199] Normality of the plasma concentrations of tenofovir, emtricitabine and raltegravir was tested using Kolmogorov-Smirnov test. Significance of SNPs for tenofovir, emtricitabine and raltegravir PK were performed separately for week 4 and week 24 sample collection. A univariate analysis was performed to identify the variables associated with tenofovir, emtricitabine and raltegravir plasma concentrations. The variables that had an association (P value < 0.15) were included in the multivariate stepwise regression. A P value of < 0.05 was considered

statistically significant for the multivariate analysis. Due to changes in drug concentrations with time, time post-dose was included in the multivariate analysis *a priori*. All statistical analysis was conducted using IBM SPSS Statistics v. 22.0.

3.3 Results

3.3.1 Patient Characteristics and Genotyping

Data were collected from 674 patients, of which 332 were randomised to the raltegravir arm and 342 were randomised to the TDF / emtricitabine arm of the study. Samples were excluded from tenofovir, emtricitabine and raltegravir analysis due to missing data for demographics, recorded time post-dose, genotype or drug plasma concentrations. Those with post-dose > 30 hours and drug concentrations below LLQ were also omitted from the analysis. All groups used for analysis had a greater proportion of males to females. Patient demographics and clinical characteristics have been described in **Table 3.2**. The allele frequencies for the tested SNPs are described in **Table 3.3.** All genotypes were in Hardy-Weinberg equilibrium except for *SLC22A6* 728 C>T (rs11568626) at week 4 and 24 for the analysis of raltegravir.

Table 3.2 Clinical characteristics and demographics of patients included in the pharmacogenetic analysis of NEAT001/ANRS143 pharmacokinetic sub study stratified by study drug and week of sample collection (data expressed in median (range) unless stated otherwise

Parameter	Tenofovir		Emtricitabine		Raltegravir	
	Week 4	**Week 24**	**Week 4**	**Week 24**	**Week 4**	**Week 24**
Included for analysis (n)	230	254	267		245	217
Age (years)	39 (18-76)	39 (18-76)	39 (18-76)	38 (18-76)	36 (19-70)	36 (19-70)
Sex [n (%)]						
Male	203 (88.3)	225 (88.6)	233 (87.3)	239 (87.5)	217 (88.6)	191 (88)
Female	27 (11.7)	28 (11)	33 (12.4)	33 (12.1)	28 (11.4)	26 (12)
Transgender	0	0	1 (4)	1 (4)	0	0
Weight at randomisation (kg)	72 (44.3 - 124.5)	72 (44.3 - 124.5)	72 (44.3 - 124.5)	73 (44.3 - 124.5)	71.3 (41-135)	72 (41-135)
Height (cm)	175.5 (154 - 202)	176 (154 - 199)	176 (154-202)	176 (154-202)	176 (153-204)	176 (153-204)

Table 3.3. Allele frequencies for SNPs investigated for pharmacogenetic analysis; n (%)

	Week 4	Week 24
Tenofovir		
Number of patients	230	254
***ABCC2* 24C>T (rs717620)**		
CC	162 (70.4)	175 (68.9)
CT	60 (26.1)	70 (27.6)
TT	8 (3.5)	9 (3.5)
***ABCC2* 1249 G>A (rs2273697)**		
GG	145 (63)	152 (59.8)
GA	75 (32.6)	89 (35)
AA	10 (4.3)	13 (5.1)
***ABCC10* 526G>A (rs9349256)**		
GG	84 (36.5)	90 (35.4)
GA	102 (44.3)	122 (48)
AA	44 (19.1)	42 (16.5)
***ABCC10* 2843 T>C (rs2125739)**		
TT	131 (57)	143 (56.3)
TC	87 (37.8)	96 (37.8)
CC	12 (5.2)	15 (5.9)
Emtricitabine		
Number of patients	267	273
***SLC47A1* G>A (rs2289669)**		
GG	93 (34.8)	86 (31.5)
AG	137 (51.3)	147 (53.8)
AA	37 (13.9)	40 (14.7)

Raltegravir		
Number of patients	245	217
***SLC22A6* 453 G>A (rs4149170)**		
GG	180 (73.5)	156 (71.9)
AG	57 (23.3)	54 (24.9)
AA	8 (3.3)	7 (3.2)
***SLC22A6* 728 C>T (rs11568626)**		
CC	239 (97.6)	211 (97.2)
CT	5 (2)	5 (2.3)
TT	1 (0.4)	1 (0.5)
***UGT1A1**28* (rs8175347)**		
*1/*1, *1/*36 (moderate enzyme activity)	102 (41.6)	89 (41)
*1/*28, *28/*26, *26/*27 (reduced enzyme activity)	106 (43.3)	95 (43.8)
*28/*28 (low enzyme activity)	37 (15.1)	33 (15.2)

3.3.2 Influence of SNPs on Tenofovir Plasma Concentrations

According to the Kolmogorov-Smirnov test, the plasma concentrations of tenofovir were not equally distributed. Hence, log concentrations of tenofovir were used for analysis.

At week 4 (**Table 3.4**), a univariate analysis showed time of post-dose blood collection ($p < 0.0001$), *ABCC2* 1249G>A ($p = 0.042$), *ABCC10* 526G>A ($p = 0.005$) and *ABCC10* 2843T>C ($p = 0.042$) to be statistically significant. *ABCC2* 24C>T did not influence ($p = 0.201$) tenofovir concentrations (**Figure 3.1**). However, on performing multivariate linear regression, *ABCC2* 1249G>A ($p = 0.272$), *ABCC10* 526G>A ($p = 0.067$) and *ABCC10* 2843T>C ($p = 0.236$) did not show an influence on tenofovir plasma concentrations (**Figure 3.2**).

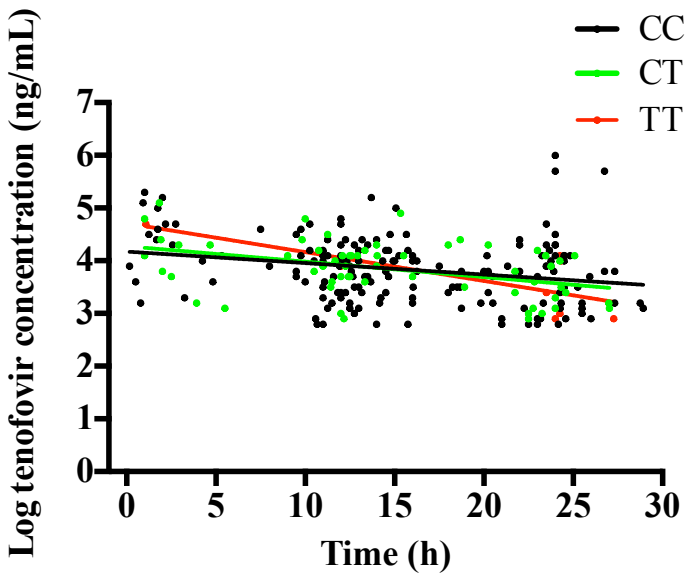

Figure 3.1. Influence of *ABCC2* 24C>T on $C_{0\text{-}24}$ tenofovir concentrations at week 4 for *ABCC10* 2843T>C. Solid lines represent mean tenofovir plasma concentrations. Univariate analysis showed no significant associations between *ABCC2* 24C>T and $C_{0\text{-}24}$ tenofovir concentrations.

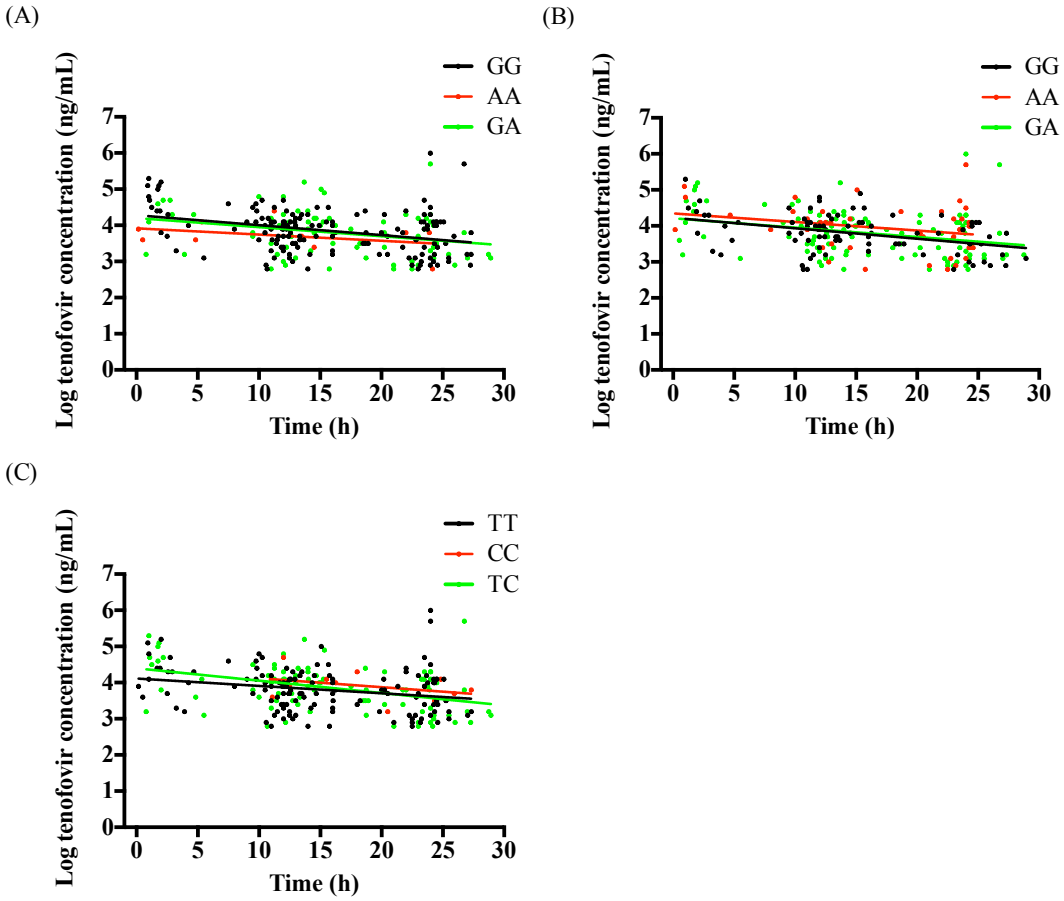

Figure 3.2. Influence of (A) *ABCC2* 1249G>A, (B) *ABCC10* 526G>A, (C) *ABCC10* 2843T>C on $C_{0\text{-}24}$ tenofovir concentrations at week 4. Solid lines represent mean tenofovir plasma concentrations. Multivariate analysis showed no significant associations between *ABCC2* 1249G>A, *ABCC10* 526G>A, and *ABCC10* 2843T>C and $C_{0\text{-}24}$ tenofovir concentrations.

Table 3.4. Association of patient characteristics and SNPs in *ABCC2* and *ABCC10* with log plasma tenofovir concentrations in patients enrolled in NEAT001/ANRS143. Variables that were significant after univariate analysis (P < 0.15) were considered for multivariate regression analysis.

Patient Characteristics	**Week 4**				**Week 24**			
	Univariate linear regression		**Multivariate linear regression**		**Univariate linear regression**		**Multivariate linear regression**	
	β	**P value**	**β**	**P value**	**β**	**P value**	**β**	**P value**
Age	0.091	0.175	-	-	0.072	0.246	-	-
Sex	-0.032	0.666	-	-	0.033	0.63	-	-
Weight at randomisation	0.237	0.573	-	-	0.058	0.776	-	-
Weight at blood collection	-0.272	0.521	-	-	-0.106	0.601	-	-
Height	-0.088	0.313	-	-	-0.064	0.421	-	-
Time of post-dose blood collection	-0.355	**< 0.0001**	-0.025	**< 0.0001**	-0.425	**< 0.0001**	-0.419	**< 0.0001**
***ABCC2* 24C>T (rs717620)**	-0.083	0.201	-	-	0.046	0.442	-	-
***ABCC2* 1249G>A (rs2273697)**	-0.133	**0.042**	-0.069	0.272	-0.014	0.821	-	-
***ABCC10* 526G>A (rs9349256)**	0.199	**0.005**	0.116	0.067	0.062	0.342	-	-
***ABCC10* 2843 T>C (rs2125739)**	0.139	**0.042**	0.074	0.246	0.044	0.482	-	-

At week 24, time of post-dose blood collection was the only variable that had a significant effect on the plasma concentrations of tenofovir (**Table 3.4**). The tested SNPs, *ABCC2* 24C>T (p = 0.442), *ABCC2* 1249G>A (p = 0.821), *ABCC10* 526G>A (p = 0.342) and *ABCC10* 2843T>C (p = 0.482), did not influence tenofovir concentrations (**Figure 3.3**).

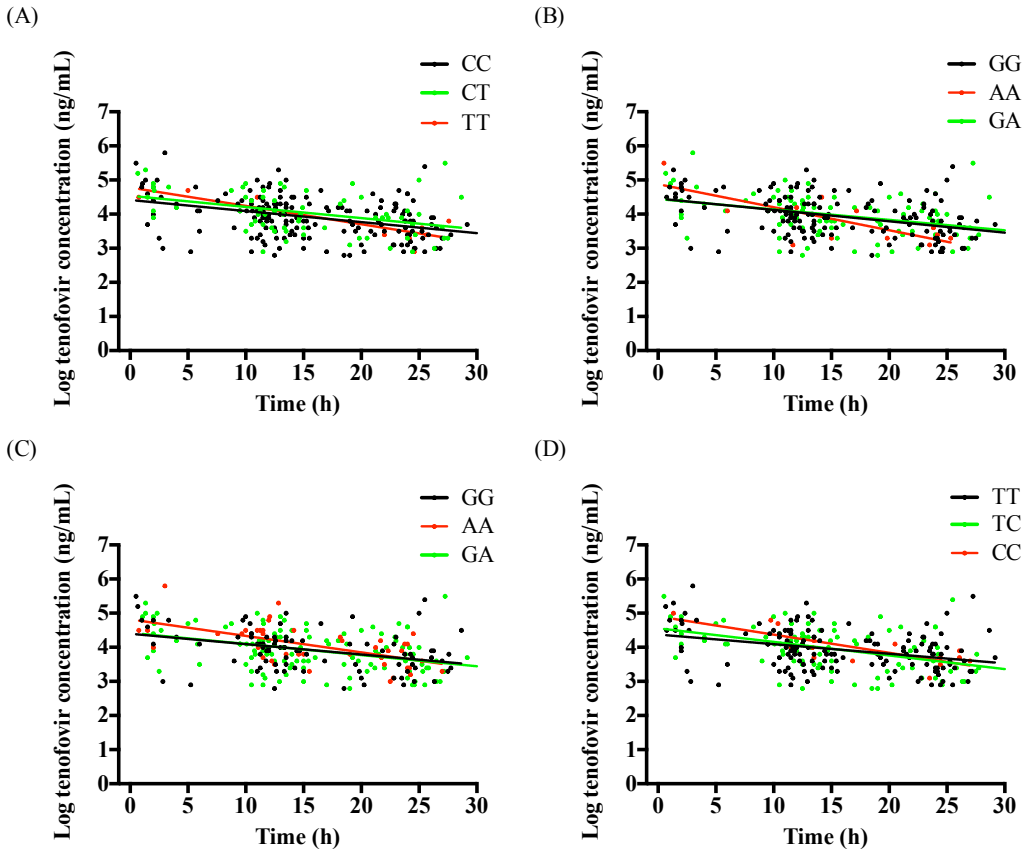

Figure 3.3. Influence of (A) *ABCC2* 24C>T, (B) *ABCC2* 1249G>A, (C) *ABCC10* 526G>A and (D) *ABCC10* 2843T>C on $C_{0\text{-}24}$ tenofovir concentrations at week 24. Solid lines represent mean tenofovir plasma concentrations. Univariate analysis showed no significant associations between SNPs and $C_{0\text{-}24}$ tenofovir concentrations.

3.3.3 Influence of SNPs on Emtricitabine Plasma Concentrations

The Kolmogorov-Smirnov test showed that he emtricitabine concentrations were not normally distributed. Hence, log concentrations of emtricitabine was used for analysis.

At week 4, a univariate linear regression showed that the tested variable *SLC47A1* G>A (**Figure 3.4**) did not have any significant effect (p = 0.502) on emtricitabine concentrations (**Tabl3 3.5**).

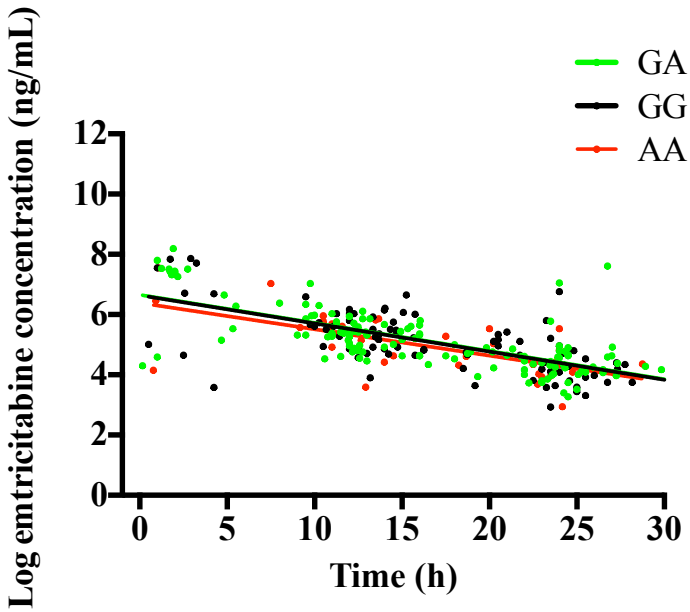

Figure 3.4. Influence of *SLC47A1* G>A on $C_{0\text{-}24}$ emtricitabine concentrations at week 4. Solid lines represent mean emtricitabine plasma concentrations. Univariate analysis did not show significant associations between *SLC47A1* G>A and $C_{0\text{-}24}$ emtricitabine concentrations.

A univariate linear regression at week 24 showed that weight at the time of blood collection ($p = 0.053$), time of post-dose blood collection ($p < 0.0001$) and *SLC47A1* G>A (**Figure 3.5**) were significantly associated with emtricitabine plasma concentrations. However, a multivariate linear regression did not confirm any significance (**Table 3.5**).

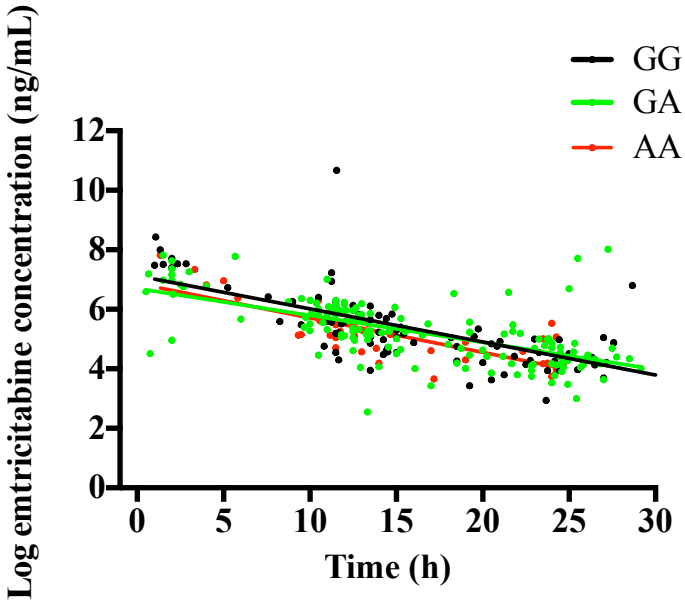

Figure 3.5. Influence of *SLC47A1* G>A on $C_{0\text{-}24}$ emtricitabine concentrations at week 24. Solid lines represent mean emtricitabine plasma concentrations. Multivariate analysis did not show significant associations between *SLC47A1* G>A and $C_{0\text{-}24}$ emtricitabine concentrations ($p = 0.07$).

Table 3.5. Association of patient characteristics and SNPs in *SLC47A1 G>A* with log plasma emtricitabine concentrations in patients enrolled in NEAT001/ANRS143. Variables that were significant after univariate analysis (P < 0.15) were considered for multivariate regression analysis.

Patient Characteristics	**Week 4**				**Week 24**			
	Univariate linear regression		**Multivariate linear regression**		**Univariate linear regression**		**Multivariate linear regression**	
	β	**P value**	**β**	**P value**	**β**	**P value**	**β**	**P value**
Age	0.044	0.344	-	-	0.086	**0.075**	0.054	0.24
Sex	-0.043	0.429	-	-	0.023	0.67	-	-
Weight at randomisation	0.013	0.965	-	-	0.195	0.21	-	-
Weight at blood collection	-0.082	0.784	-	-	0.195	**0.053**	-0.066	0.152
Height	0.039	0.526	-	-	0.058	0.361	-	-
Time of post-dose blood collection	-0.608	**< 0.0001**	-0.691	**< 0.0001**	-0.664	**< 0.0001**	0.658	**< 0.0001**
***SLC47A1* G>A (rs2289669)**	-0.031	0.502	-	-	-0.091	**0.049**	-0.083	0.07

3.3.4 Influence of SNPs on Raltegravir Plasma Concentrations

According to the Kolmogorov-Smirnov test, the plasma concentrations of raltegravir was not equally distributed. Hence, log concentrations of raltegravir was used for analysis.

At week 4 (**Table 3.6**), a univariate linear regression found baseline weight ($p = 0.021$), weight at the time of blood sample collection ($p = 0.006$) and time of post-dose blood sample collection ($p < 0.0001$) to be significant. The tested polymorphisms *SLC22A6* 453G>A ($p = 0.139$), *SLC22A6* 728C>T ($p = 0.24$) and *UGT1A1**28 ($p = 0.301$) had no significant effect on raltegravir plasma concentrations (**Figure 3.6**). Multivariate regression confirmed the effect of baseline weight ($p = 0.027$), weight at the time of blood sample collection ($p = 0.008$) and time of post-dose blood sample collection ($p < 0.0001$) to have a statistically significant effect on the plasma concentrations of raltegravir.

Table 3.6. Association of patient characteristics and SNPs in *SLC22A6* and *UGT1A1* with log plasma raltegravir concentrations in patients enrolled in NEAT001/ANRS143. Variables that were significant after univariate analysis (P < 0.15) were considered for multivariate regression analysis.

Patient Characteristics	**Week 4**				**Week 24**			
	Univariate linear regression		**Multivariate linear regression**		**Univariate linear regression**		**Multivariate linear regression**	
	β	**P value**	**β**	**P value**	**β**	**P value**	**β**	**P value**
Age	-0.056	0.365	-	-	-0.022	0.726	-	-
Sex	-0.049	0.498	-	-	-0.093	0.926	-	-
Weight at randomisation	0.853	**0.021**	0.812	**0.027**	0.241	0.183	-	-
Weight at blood collection	-1.01	**0.006**	-0.985	**0.008**	-0.318	**0.08**	-0.073	0.217
Height	0.011	0.889	-	-	0.046	0.57	-	-
Time of post-dose blood collection	-0.385	**< 0.0001**	-0.367	**< 0.0001**	-0.531	**< 0.0001**	-0.506	**< 0.0001**
***SLC22A6* 453G>A (rs4149170)**	-0.096	0.139	-	-	0.036	0.581	-	-
***SLC22A6* 728C>T (rs11568626)**	-0.075	0.24	-	-	-0.006	0.929	-	-
***UGT1A1**28 (rs8175347)**	0.062	0.301	-	-	0.124	**0.038**	0.115	0.05

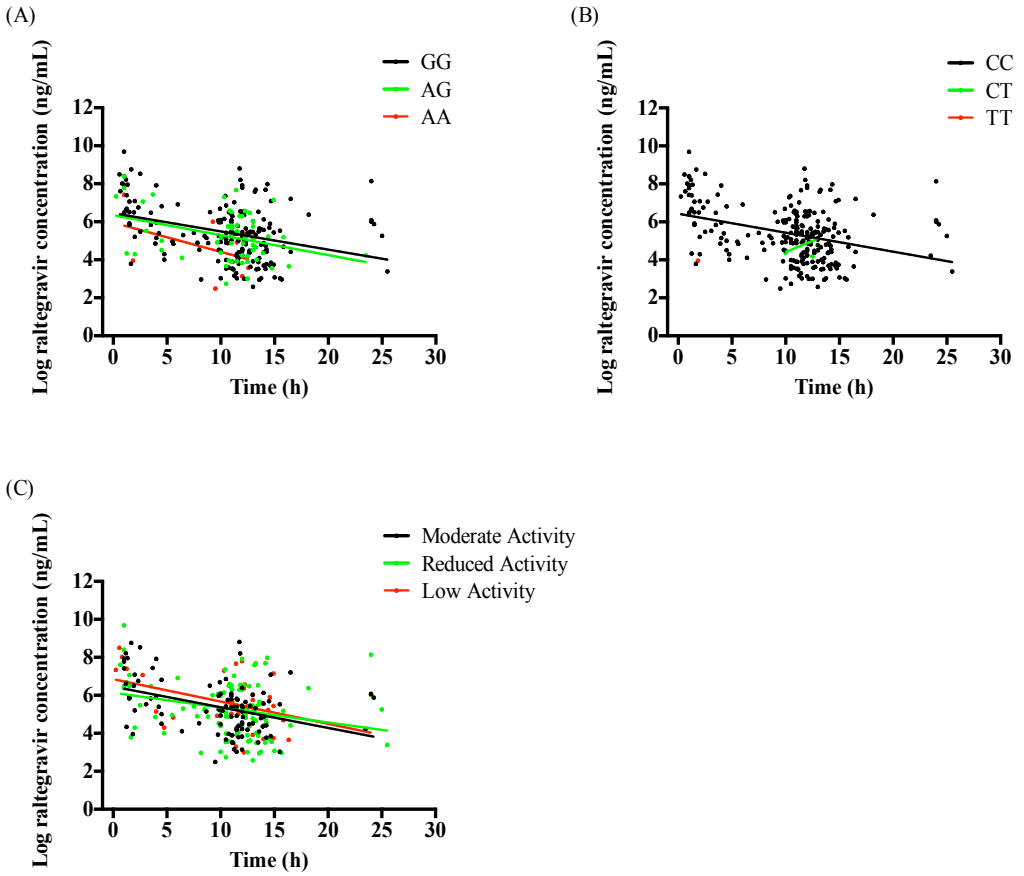

Figure 3.6. Influence of (A) *SLC22A6* 453G>A, (B) *SLC22A6* 728C>T, (C) *UGT1A1**28 on $C_{0\text{-}24}$ raltegravir concentrations at week 4. Solid lines represent mean raltegravir plasma concentrations. Univariate analysis did not show significant associations between the SNPs and $C_{0\text{-}24}$ raltegravir concentrations.

At week 24, weight at the time of blood sample collection (p = 0.08), time of post-dose blood sample collection (p < 0.0001) and *UGT1A1**28 (p = 0.038) were significant by a univariate linear regression. *SLC22A6* 453G>A (p = 0.0.581) and *SLC22A6* 728C>T (p = 0.929) did not influence raltegravir plasma concentrations (**Figure 3.7**). On performing a multivariate regression, time of post-dose blood sample collection (p < 0.0001) was the only variable to have a statistically significant effect on raltegravir plasma concentration. Although not significant, a P value of 0.05 and β of 0.115 shows a trend of higher raltegravir plasma concentrations in patient in with *UGT1A1**28 (**Figure 3.8**).

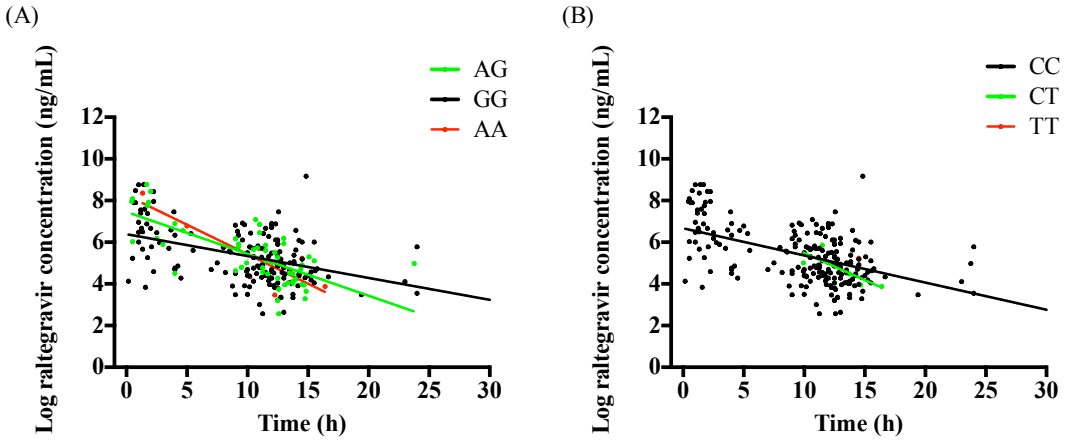

Figure 3.7. Influence of (A) *SLC22A6* 453G>A, (B) *SLC22A6* 728C>T on $C_{0\text{-}24}$ raltegravir concentrations at week 24. Solid lines represent mean raltegravir plasma concentrations. Univariate analysis did not show significant associations between the SNPs and $C_{0\text{-}24}$ raltegravir concentrations.

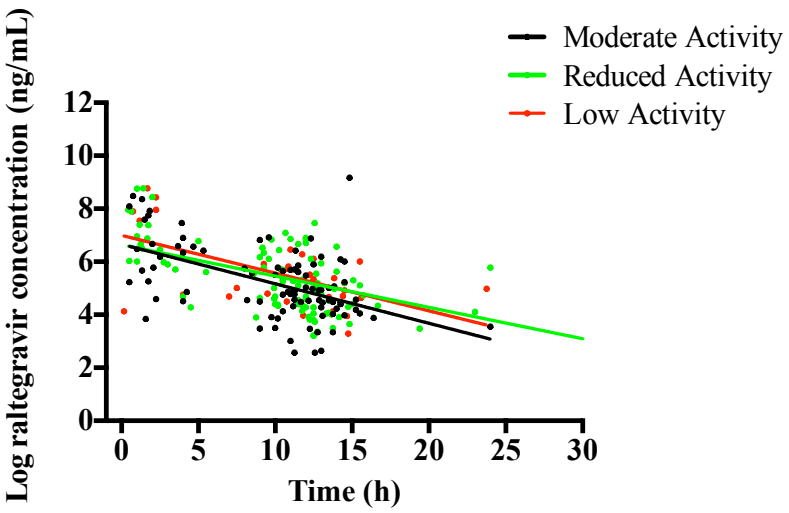

Figure 3.8. Influence of *UGT1A1**28 on $C_{0\text{-}24}$ raltegravir concentrations at week 24. Solid lines represent mean raltegravir plasma concentrations. Multivariate analysis did not show significant associations between the *UGT1A1**28 ($p = 0.05$) and $C_{0\text{-}24}$ raltegravir concentrations.

3.4 Discussion

All genotypes were in Hardy-Weinberg equilibrium except for *SLC22A6* 728 C>T (rs11568626) at week 4 and 24 for the analysis of raltegravir. This indicates missingness of genotypes due to chance, genotyping assay errors or clinical biases. Hence, the results from these genotypes should be interpreted with caution while applying to the general populations.

This is the first study to investigate the effect of clinically relevant SNPs on tenofovir, emtricitabine and raltegravir, in a large population. TDF is transported in the proximal tubules by the ABCC4 (MRP4),[227] ABCC11 (MRP8),[228] ABCC10,[229] OAT1 and OAT3.[230] It is excreted unchanged by the kidneys and this gives rise to renal tubular toxicity.[225] Genetic polymorphisms in the transporters could lead to development or exacerbation of tubular toxicity. Although, there is no proof of tenofovir being a substrate of ABCC2, the effect of *ABCC2* polymorphisms have been demonstrated in the past. The *ABCC2* 24C>T (rs717620) and *ABCC2* 1249G>A (rs2273697) were found to be protective for kidney toxicity in Japanese populations.[244] This study, however, failed to show a significant effect of 14 SNPs in *ABCC4, ABCC10, SCL22A6,* and *ABCB1* genes on tenofovir induced kidney toxicity. It has been suggested that endogenous substrates of ABCC2 either exacerbate or compete with tenofovir transport by ABCC4. Alternatively, *ABCC2* genotype may be in linkage disequilibrium with other polymorphisms that code for factors that increase toxicity.[245] Pushpakom *et al*. demonstrated the transport of tenofovir by the ABCC10 (MRP-7) transporter and demonstrated the association of *ABCC10* 526G>A (rs9349256) and *ABCC10* 2843T>C (rs2125739) with kidney toxicity.[229] This was demonstrated in vitro using HEK-293-ABCC10 cell lines.

We investigated the effect of *ABCC2* 24C>T and *ABCC2* 1249G>A polymorphisms on tenofovir plasma concentrations and found no significant changes. Similarly, no associations of *ABCC10* 526G>A and *ABCC10* 2843T>C with tenofovir plasma concentrations were evident. To further investigate the clinical impact of SNPs, it is important to analyse the associations of the polymorphisms with adverse events.

A common limitation in such studies is the lack of investigations into the intracellular concentrations of drugs that can influence drug pharmacodynamics.[246] Liptrott *et al.* demonstrated an interaction between tenofovir and nevirapine at the intracellular level, that cannot be explained by changes in the plasma concentrations. This indicates involvement of factors such as transporters at the cellular site and intracellular binding in tenofovir disposition.[247] Polymorphisms in these transporters and proteins can influence the efficacy and kidney toxicity of tenofovir, warranting further investigations.

Emtricitabine is excreted mostly unchanged[236] and is a substrate of the MATE1 transporter found in the kidney.[237] The *SCL47A1* G>A (rs2289669) is found to be associated with response to treatment and reduction in HbA1C in patients receiving metformin.[248] It is postulated that *SCL47A1* G>A results in reduced function or expression of MATE1.[249] Our findings showed no associations between *SCL47A1* G>A and emtricitabine plasma concentrations.

Raltegravir is metabolized by glucuronidation by the *UGT1A1* isoform.[240] Polymorphisms in the *UGT1A1* gene have been shown to influence the PK of its substrates. For example, *UGT1A1**28 and *UGT1A1**6 are associated with severe toxicities in patients undergoing irinotecan based chemotherapy.[250] UGT1A1 is

responsible for the conjugation of bilirubin and *UGT1A1**28 results in higher concentrations of unconjugated bilirubin known as Gilbert syndrome.[251]

The investigations of the impact of SNPs in *UGT1A1* on raltegravir have given variable results. Wenning *et al*. showed that the plasma concentrations of raltegravir were significantly higher in patients with the *UGT1A1**28 allele.[168] Whereas, Hirano *et al*. did not establish any significant differences in the raltegravir plasma concentrations due to *UGT1A1* polymorphisms.[252] Recently, a study conducted on 104 HIV infected individuals showed that *UGT1A1**28 was associated with significantly higher raltegravir plasma concentrations.[253] Additionally, the *UGT1A1**6 polymorphism was also shown to affect raltegravir plasma concentrations.[254]

Our study did not show associations between *UGT1A1**36 and *UGT1A1**37 and raltegravir plasma concentrations. Multivariate regression analysis did not show associations between *UGT1A1**28 and raltegravir plasma concentration. However, a P value of 0.5 with β of 0.115 indicates that although not statistically significant, *UGT1A1**28 may result in higher raltegravir plasma concentration.

Interestingly, at week 4, the weight of the patient at baseline ($p = 0.027$) and at the time of week 4 blood sample collection ($p = 0.008$) was associated with raltegravir plasma concentrations. In the past, raltegravir concentrations shown to be dependent on patient's body weight[255] and use of raltegravir may be associated with weight gain and lipodystrophy.[256] The relationship between raltegravir plasma concentrations and body weight need to be explored in more detail as it may give further insight into inter and intra patient variability[257] that is observed during raltegravir administration.

The limitations in Chapter 2 regarding the use of NONMEM as a better analytical tool, lack of co-relation with pharmacodynamic data and difference in the methodologies in our studies compared to the ones in literature are relevant for this study since they both involve the NEAT001/ANRS143 study and a similar methodology for analysis.

In conclusion, the SNPs tested for tenofovir, emtricitabine and raltegravir did not influence the plasma concentrations. Although statistically insignificant, *UGT1A1**28 was associated with raltegravir plasma concentrations. Weight of the patients also influenced raltegravir plasma concentrations and this relationship needs to be explored in more detail. Moreover, an analysis of the SNPs on drug PD and adverse events is warranted.

Chapter 4

An investigation of HIV Protease Inhibitors Binding to Plasma Proteins in vitro: Possible Implications for Pharmacokinetics

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4.4 Discussion

4.1 Introduction

The WHO 2015 guidelines recommend initiation of multidrug ART for all HIV infected individuals regardless of the WHO clinical stage or $CD4^+$ T-cell count.[258] ART is often combined with other drugs used to treat OIs or other concomitant illnesses, which commonly results in DDIs.[259] HIV PIs can either act as perpetrators or victims[138] and a better understanding of the mechanisms that underpin pharmacokinetic DDIs can improve understanding and prediction, which can be important for clinical management.

On reaching the systemic circulation, many drugs bind to circulating plasma proteins, such as albumin (present at ~40 mg/mL) and AAG (present at ~0.7 mg/mL),[260] and to a lesser extent, globulins and lipoproteins.[152] Protein-bound drug remains in the blood circulation whereas unbound drug distributes into cells and tissues to exert the therapeutic effect (**Figure 1.7**).[261] Changes in concentration of plasma proteins and the percentage of bound drug can affect PK via an impact upon the clearance (particularly for drugs with a low hepatic extraction ratio)[262] or by altering the distribution into tissues and cells.[263] PBPK models depend on accurate predictions of volume of distribution and clearance, making plasma protein binding a critical feature of the models.[264]

Conditions such as myocardial infarction and malignancies,[265] liver and kidney impairment,[266,267] depression,[268] pregnancy,[269] concomitant administration of contraceptive steroids[270] as well as surgical procedures[271] and age[260] have all been shown to influence plasma AAG and albumin concentrations. Therefore, any of these factors may influence the pharmacokinetic profile of highly bound drugs.

When co-administered with another drug that has a higher binding affinity to plasma proteins, drugs can be displaced from the protein, causing an increase in the unbound percentage. This could result in an increase in C_{max}, potentially causing toxicity and warranting dose adjustment.[272] Moreover, higher unbound concentrations may increase the clearance resulting in a drop in C_{min} below MEC.[273] Therefore, in some cases protein binding can be important for treatment response.

Inconsistent reports have been published with regards to the binding affinity of HIV PIs.[274,275] These discrepancies warrant further investigation of the affinity and the binding characteristics of the HIV PIs to understand their effects on drug distribution and PK. In this study, the binding of commonly used PIs to both AAG and albumin was assessed using rapid equilibrium dialysis (RED). To better understand the consequences, darunavir displacement by model binding displacers was assessed in buffer containing AAG and albumin separately and in combination.

4.2 Methods

4.2.1 Reagents

Darunavir and atazanavir were obtained from Selleckchem (USA). Ritonavir and lopinavir were acquired from LGM Pharma (USA). Quinidine, sodium salicylate, ibuprofen, sodium valproate, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), AAG, albumin, and PBS tablets were obtained from Sigma-Aldrich (USA). Acetonitrile (ACN) was obtained from Fluka analytical (South Africa). Pooled human plasma was obtained from the National Health Service (NHS, UK). RED plates and tube 8 KDa MWCO inserts were obtained from Thermo Fisher Scientific (USA).

4.2.2 Study Design

The plasma protein binding of darunavir, atazanavir, lopinavir and ritonavir was analysed in pooled human plasma. Their displacement from the plasma proteins was assessed by measuring the increase in binding percentage in the presence of the known AAG[276] and albumin displacers[277] (**Table 4.1**). Displacement was also measured in the presence of all the displacers (quinidine + sodium salicylate + ibuprofen + sodium valproate) to assess the overall impact of displacement from AAG and albumin. Similar experiments were conducted to check the displacement of darunavir by AAG and albumin displacers in buffer containing AAG and albumin separately and in combination.

Table 4.1. Known displacers for AAG and albumin plasma proteins

Plasma Protein	Binding Site	Displacer
AAG[276]		Quinidine
Albumin[277]	Sudlow I	Sodium Salicylate
	Sudlow II	Ibuprofen
	Bilirubin	Sodium Valproate

4.2.3 Rapid Equilibrium Dialysis (RED)

Protein binding was assessed using RED. Stock solutions of PIs and displacers were made in DMSO. Sodium valproate stocks were prepared in water.

Dialysis tubes were soaked according to manufacturer's instructions in 10 % isopropyl alcohol (IPA) for 10 minutes to remove the glycerine coating from the dry dialysis membrane. The dialysis device was rinsed and soaked in distilled water for a further 20 minutes to wash off the IPA and the glycerine residue.

Human plasma at 37°C was spiked with C_{max} concentrations of darunavir (11.86 μM, 6.5 μg),[278] atazanavir (6.24 μM, 4.4 μg),[279] lopinavir (22.26 μM, 14 μg)[279] and ritonavir (20.5 μM, 14.8 μg).[280] The spiked plasma was used to prepare aliquots for experimentations, containing AAG displacer quinidine (100 μM) and albumin displacers sodium salicylate (200 μM), ibuprofen (50 μM) and sodium valproate (500 μM) (**Table 4.2**).

Table 4.2. Experimental conditions used to analyse the displacement of PIs using AAG and albumin displacers in plasma and buffer containing AAG, albumin, and both

Protease Inhibitor	**Buffer**			
	Plasma	AAG	Albumin	AAG + Albumin
Darunavir	Darunavir Darunavir + Quinidine Darunavir + Sodium Salicylate Darunavir + Ibuprofen Darunavir + Sodium Valproate Darunavir + All displacers	Darunavir Darunavir + Quinidine	Darunavir Darunavir + Sodium Salicylate Darunavir + Ibuprofen Darunavir + Sodium Valproate	Darunavir Darunavir + Quinidine Darunavir + Sodium Salicylate Darunavir + Ibuprofen Darunavir + Sodium Valproate Darunavir + All displacers
Atazanavir	Atazanavir Atazanavir + Quinidine Atazanavir + Sodium Salicylate Atazanavir + Ibuprofen Atazanavir + Sodium Valproate Atazanavir + All displacers			
Lopinavir	Lopinavir Lopinavir + Quinidine Lopinavir + Sodium Salicylate Lopinavir + Ibuprofen Lopinavir + Sodium Valproate Lopinavir + All displacers			
Ritonavir	Ritonavir Ritonavir + Quinidine Ritonavir + Sodium Salicylate Ritonavir + Ibuprofen Ritonavir + Sodium Valproate Ritonavir + All displacers			

Additionally, the displacement experiment was repeated for darunavir (11.86 μM) in buffer containing AAG (0.7 mg/mL), albumin (40 mg/mL) at levels found in healthy humans[260] with AAG and albumin displacers (**Table 4.2**). These experiments were performed to confirm that the displacement of PIs in plasma by the displacers were specifically from AAG and albumin. Darunavir was chosen for these additional experiments as it is the only PI that has not been shown to bind to albumin.[275]

A volume of 500 μL plasma from each condition was placed in the donor compartment of the RED inserts. An aliquot (750 μL) of PBS was placed in the receiver compartment. The average pore size of semi-permeable membrane is less than ~2 nm (8000 Da) which prevents the transfer of plasma proteins and drugs bound to them (**Figure. 4.1 and Figure. 4.2**). The unbound drug passes freely through the semi-permeable membrane that has a molecular weight cut-off of 8K. The experiment was performed in triplicate. The RED plate was covered with paraffin film to prevent drying and incubated at 37°C on an orbital shaker at 250 rpm for four hours.

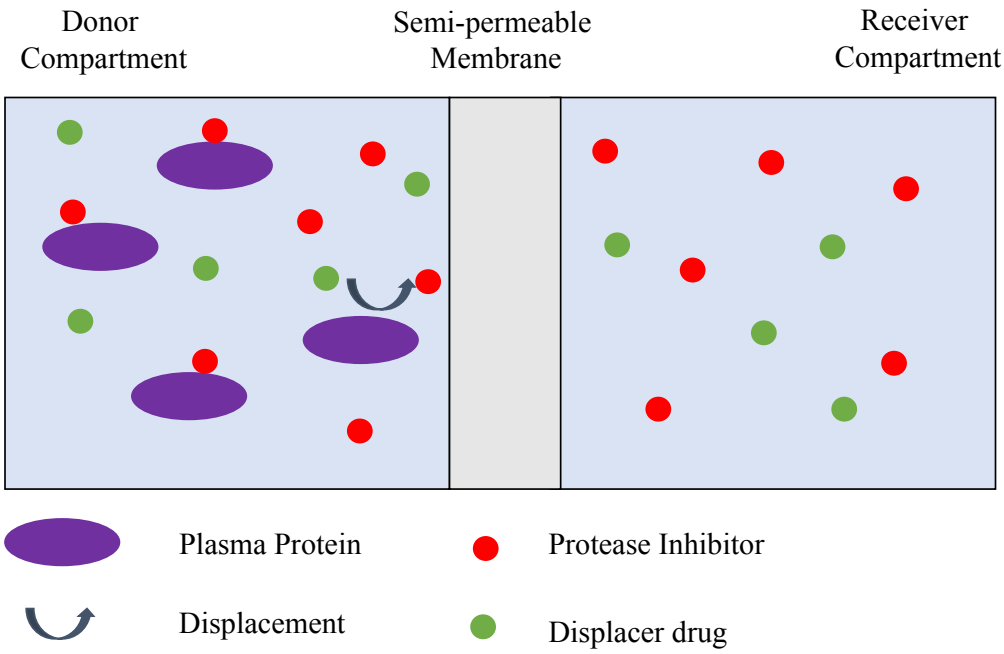

Figure 4.1. Rapid Equilibrium dialysis system with plasma proteins and drugs (bound + unbound) in the donor compartment and unbound drugs in receiver compartment

Figure 4.2. Rapid equilibrium dialysis plate and inserts. (Image from Fischer Scientific website)

4.2.4 Drug Extraction

Post-incubation, an aliquot of 200 µl of PBS was taken from the receiver chamber and was diluted with ACN to give a final ratio of PBS:ACN (60:40). A volume of 200 µl was then transferred to 300 µl chromatography vials for quantification of unbound drug concentration.

From the receiver chamber, an aliquot of 200 µl of plasma was taken and diluted with ACN to get a ratio of plasma:ACN (20:80) to induce protein precipitation. These samples were thoroughly vortexed and placed on a rotator for 60 min. Following this, the samples were centrifuged at 17,000 x *g* for 30 minutes. The supernatant fraction was then transferred to glass vials and dried at 40°C for 4 hours. The drug in the glass vials was reconstituted in water containing 40% (v/v) ACN and transferred to 300 µl chromatography vials for quantification of total drug concentration.

4.2.5 Drug Quantification

Darunavir, atazanavir, lopinavir and ritonavir were quantified by HPLC-UV using a method conducted in accordance with the FDA guidelines[281] in terms of accuracy (Mean CV <15%), precision (Mean CV < 15%) and recovery (Mean recovery > 85%) (**Table 4.3**). Method validation was completed separately for quantification from PBS buffer (receiver compartment) and plasma (donor compartment).

Chromatographic separation was performed using a Waters Atlantis T3 (4.6 x 100m, 3 µm) column (Waters, Elstree, UK) with a 10 x 4 µm Fortis C18 Guard (FortisTM Technologies Ltd, Chester, UK). A Dionex P680 HPLC pump, Dionex ASI-100 automated sampler injector and Dionex UVD170U UV detector (Thermo Fischer Ltd,

Hemel-Hempstead, UK) were used. Mobile phases C (25 mM KH2PO4, pH 3.3/orthophosphoric acid) and D (100% ACN) were used. Elution was carried out at room temperature (RT) and the flow rate was set at 1 ml/min. The chromatograms were analysed using Chromeleon software (version 6.8, Thermo Fisher Ltd). All conditions of separation except the gradient was constant for the tested HIV PIs.

For darunavir, the step-gradient elution was as follows: 70% C/30% D from 0.0 to 1.5 min, 35% C/ 65% D from 1.5 to 7.0 min, 20% C/80% D from 7.0 to 9.5 min and 70% C/30% D from 9.5 to 12.5 min.

For atazanavir, the step-gradient elution was as follows: 70% C/ 30% D from 0.0 to 1.5 min, 52.5% C/47.5% D from 1.5 to 4.0 min, 35% C/65% D from 4.0 to 6.0 min, 20% C/80% D from 6.0 to 9.5 min and 70% C/30% D from 9.5 to 12.5 min.

For lopinavir and ritonavir, the step-gradient elution was as follows: 70% C/30% D from 0.0 to 1.5 min, 40% C/60% D from 1.5 to 6.0 min, 30% C/70% D from 6.0 to 6.2 min, 20% C/80% D from 6.2 to 8.2 min and 70% C/30% D from 8.2 to 11.2 min.

Table 4.3. Parameters of HPLC-UV method validation for darunavir, atazanavir, lopinavir and ritonavir in terms of mean intra- and inter-day accuracy and precision and recovery. n = 3.

Drug	Buffer	Wavelength (nm)	Intra-day (Mean CV %)		Inter-day (Mean CV %)		Mean Recovery (%)
			Accuracy	Precision	Accuracy	Precision	
Darunavir	**PBS**	267	1	0.6	3.2	4	98.7
	Plasma	267	9.5	8.8	3.4	14.4	103
Atazanavir	**PBS**	267	0.7	2.9	0.6	1.1	91.4
	Plasma	267	4.7	4	1	10.2	100
Lopinavir	**PBS**	215	2.4	2.6	3.3	5.1	97.4
	Plasma	215	1.7	1.9	3.6	3.3	95.6
Ritonavir	**PBS**	210	4.7	1.5	0.6	5.3	91
	Plasma	210	3.8	5.8	3.5	6	89.4

A standard curve using known concentrations of drug (0.156 µM to 20,000 µM) was used for each experiment to calculate the drug concentrations in the samples. The validated methods for all the drugs meet the criteria of Mean Accuracy < 15%, Mean Precision < 15% and Mean Recovery >85%.

4.2.6 Data Analysis

The unbound drug percentage was calculated using the following formula:

$$\%\ unbound = \frac{concentration\ in\ receiver}{concentration\ in\ donor} X100$$

Normality of the data was confirmed by Shapiro-Wilk test and statistical significance was assessed using an unpaired t-test with a P value < 0.05 considered to be significant. All statistical analyses were conducted using SPSS (Version 6).

4.3 Results

4.3.1 Darunavir Displacement from Plasma Proteins in Plasma

In pooled human plasma, the unbound percentage of darunavir was 14.2 ± 0.7 % (**Figure. 4.3**). Unbound darunavir was at 17.4 ± 0.7% in the presence of AAG displacer quinidine (a difference of 3.2%, p = 0.005) and 18.2 ± 1.2% in the presence of albumin displacer sodium valproate (a difference of 4%, p = 0.014). A combination of all AAG and albumin displacers resulted in a 27.1 ± 1.8% unbound darunavir (a difference of 12.9%, p = 0.003).

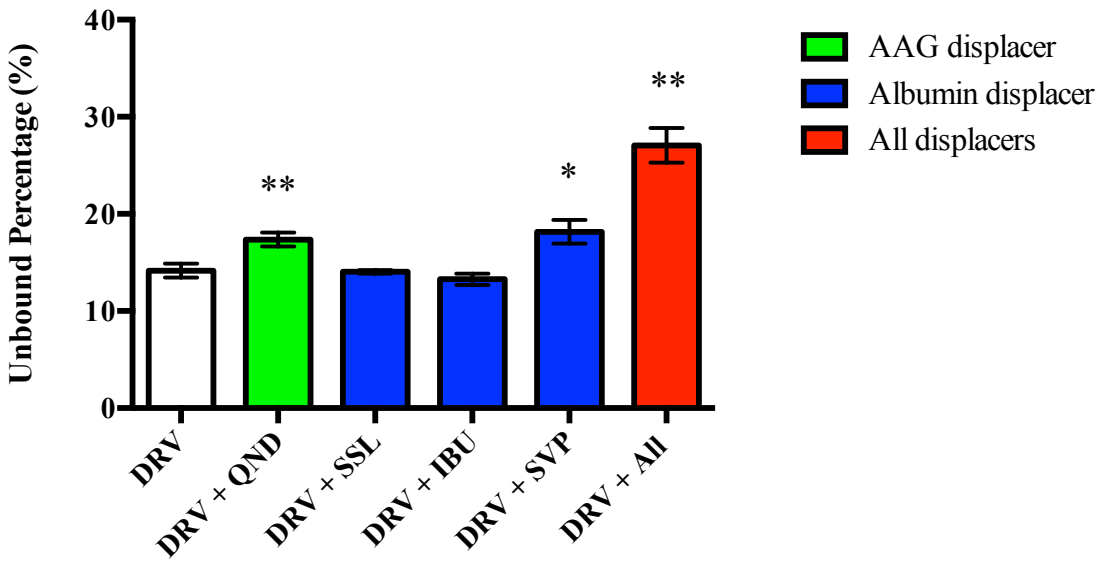

Figure 4.3. Displacement of darunavir by AAG and albumin displacers in plasma. n ≥ 3. Statistical significance by unpaired t-test. DRV, darunavir; QND, quinidine; SSL, sodium salicylate; IBU, ibuprofen; SVP, sodium valproate. P Value: *, < 0.05; **, < 0.01; ***, < 0.001; ****, ≤ 0.0001.

4.3.2 Atazanavir Displacement from Plasma Proteins in Plasma

Unbound atazanavir in human pooled plasma was 8.4 ± 0.7% of the total (**Figure. 4.4**). There was a statistically significant increase in the unbound atazanavir of 11.6 ± 0.1%, in the presence of the albumin displacer sodium valproate (a difference of 3.2%, p = 0.014). A combination of all AAG and albumin displacers increased unbound atazanavir to 13.8 ± 1.4% (a difference of 5.4%, p = 0.009).

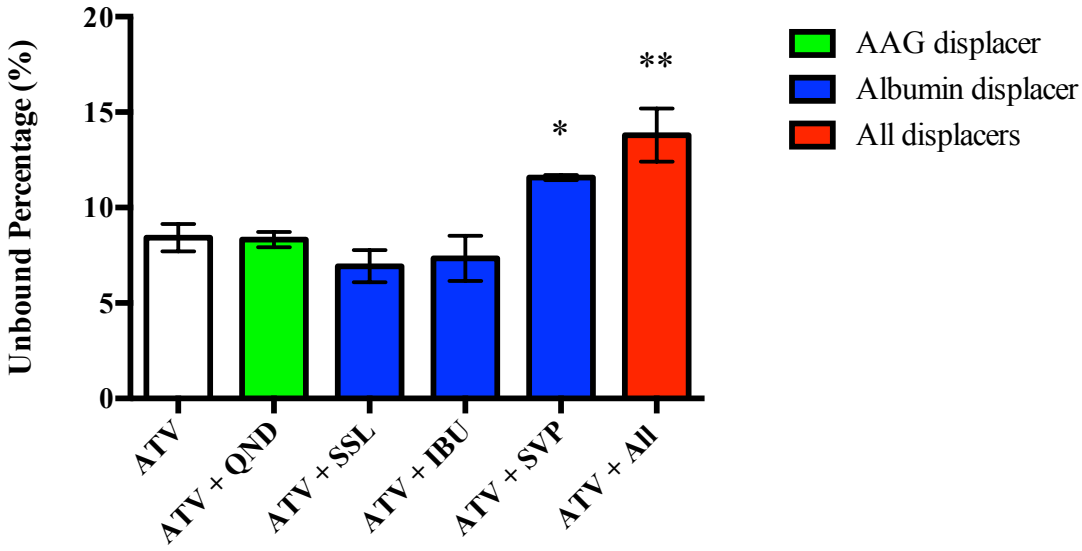

Figure 4.4. Displacement of atazanavir by AAG and albumin displacers in plasma. n ≥ 3. Statistical significance by unpaired t-test. ATV, atazanavir; QND, quinidine; SSL, sodium salicylate; IBU, ibuprofen; SVP, sodium valproate. P Value: *, < 0.05; **, < 0.01; ***, < 0.001; ****, ≤ 0.0001.

4.3.3 Lopinavir Displacement from Plasma Proteins in Plasma

In human pooled plasma, 1.1 ± 0.1% of the total lopinavir was unbound (**Figure. 4.5**). The presence of the albumin displacer sodium valproate increased unbound lopinavir to 3.0 ± 0.8% (a difference of 1.9%, p = 0.023). A combination of all AAG and albumin displacers resulted in 6.3 ± 0.5% unbound lopinavir (a difference of 5.2%, p < 0.0001).

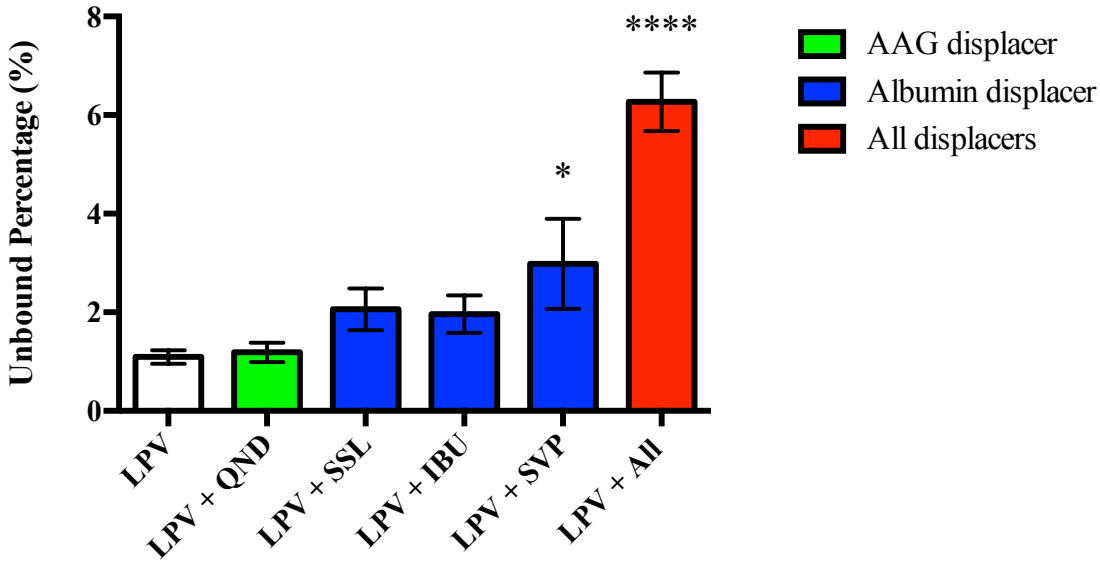

Figure 4.5. Displacement of lopinavir by AAG and albumin displacers in plasma. n ≥ 3. Statistical significance by unpaired t-test. LPV, lopinavir; QND, quinidine; SSL, sodium salicylate; IBU, ibuprofen; SVP, sodium valproate. P Value: *, < 0.05; **, < 0.01; ***, < 0.001; ****, ≤ 0.0001.

4.3.4 Ritonavir Displacement from Plasma Proteins in Plasma

Ritonavir was seen to be 0.7 ± 0.04% unbound in pooled human plasma (**Figure. 4.6**). The albumin displacer sodium valproate increased the unbound ritonavir to 2.3 ± 0.2% (a difference of 1.6%, p = 0.006). The presence of all AAG and albumin displacers resulted in a 3.0 ± 0.3% unbound ritonavir (a difference of 2.3%, p = 0.004).

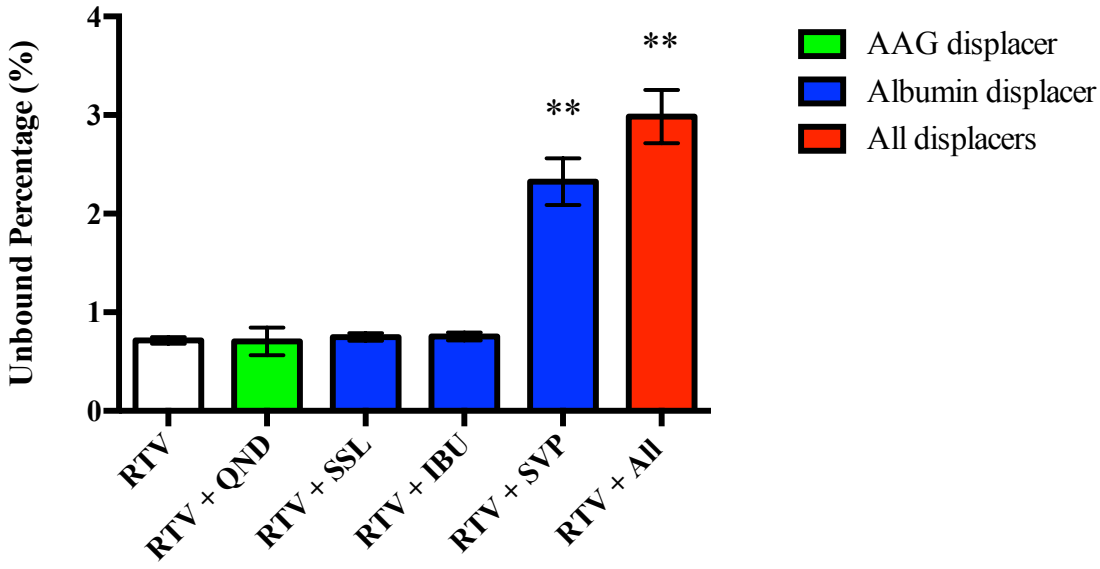

Figure 4.6. Displacement of ritonavir by AAG and albumin displacers in plasma. n ≥ 3. Statistical significance by unpaired t-test. RTV, ritonavir; QND, quinidine; SSL, sodium salicylate; IBU, ibuprofen; SVP, sodium valproate. P Value: *, < 0.05; **, < 0.01; ***, < 0.001; ****, ≤ 0.0001.

4.3.5 Darunavir Displacement in Buffer Containing AAG, Albumin and AAG + Albumin

In buffer containing AAG, the unbound percentage of darunavir was 36 ± 0.1 % (**Figure. 4.7(A)**). Unbound darunavir was measured as 45.4 ± 1.2% in the presence of the AAG displacer quinidine (a difference of 9.6%, $p = 0.005$). In buffer containing albumin, darunavir was 14.7 ± 1.8% unbound (**Figure. 4.7(B)**). The presence of the albumin displacer sodium valproate resulted in 28.2 ± 0.5% unbound darunavir (a difference of 13.5%, $p = 0.003$). In buffer containing AAG and albumin at physiological concentrations, 7.2 ± 0.3% of darunavir was unbound (**Figure. 4.7(C)**). Higher proportions of unbound darunavir (9.7 ± 0.3%) were observed in the presence of quinidine (a difference of 2.5%, $p = 0.001$) and in the presence of sodium valproate (17.4 ± 0.7%, a difference of 10.2%, $p < 0.0001$). A combination of all AAG and albumin displacers resulted in 23.6 ± 0.8% unbound darunavir (a difference of 16.4%, $p < 0.0001$).

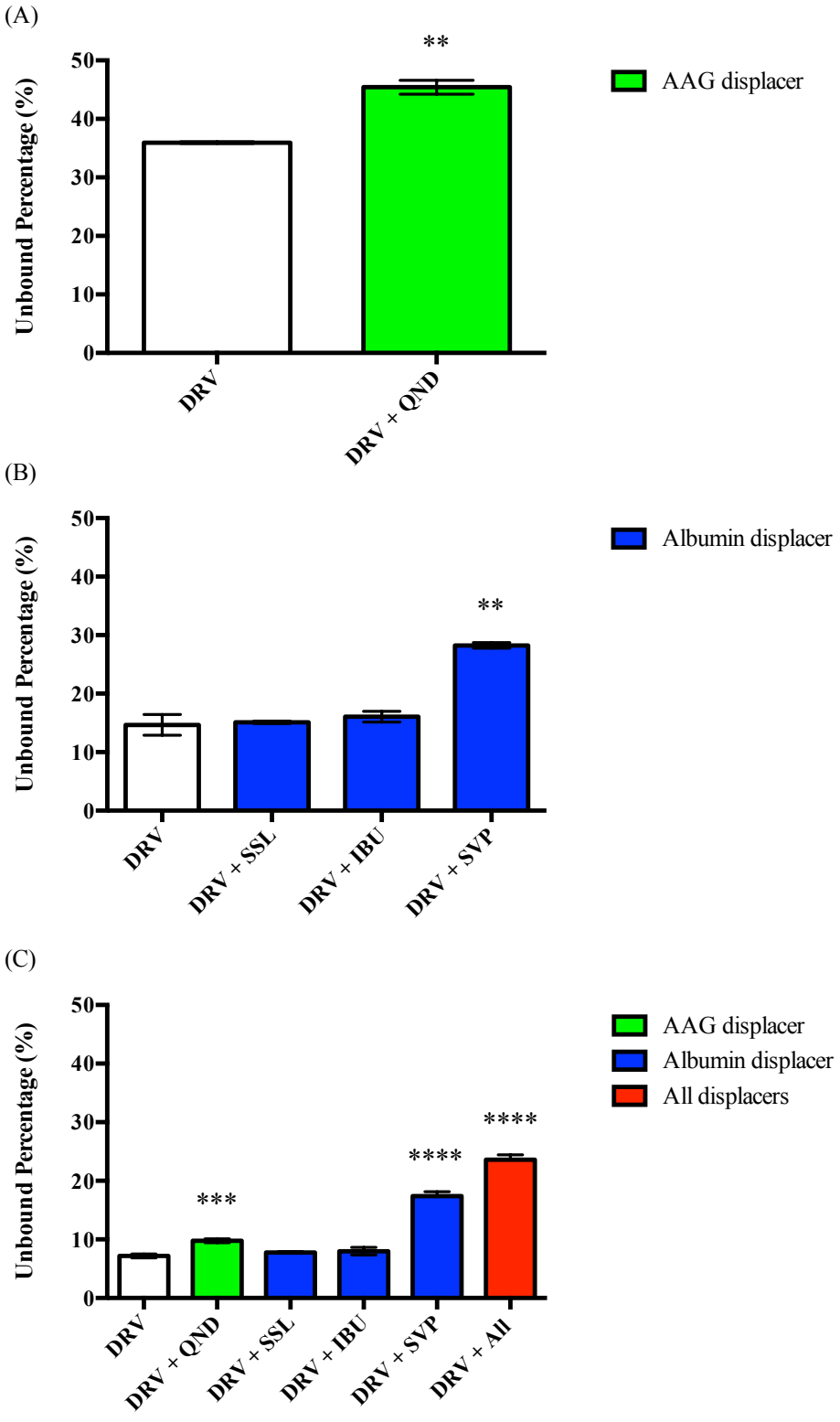

Figure 4.7. Displacement of darunavir in buffer containing AAG (A), albumin (B) and AAG + albumin. n = 3. Statistical significance by unpaired t-test. DRV, darunavir; QND, quinidine; SSL, sodium salicylate; IBU, ibuprofen; SVP, sodium valproate. P Value: *, < 0.05; **, < 0.01; ***, < 0.001; ****, ≤ 0.0001.

4.4 Discussion

In pooled human plasma, unbound darunavir was 14.2% and parallel experiments performed in buffer containing AAG, albumin and both indicated darunavir binds to both AAG and albumin. These findings differ from the values described in the literature of unbound darunavir at 3.5% with binding to AAG, which was analysed using the same methodology of RED and quantified using liquid chromatography/tandem mass spectrometry (LC-MS/MS).[275] Similarly, atazanavir was found to be 8.4% unbound, which differs from the 14% calculated *in vivo*.[282] Importantly, our study utilized plasma from healthy volunteers whereas the previous paper used plasma from HIV positive patients. Plasma protein concentration have shown to change in individuals infected with HIV.[212] Therefore, these inconsistencies may represent an important difference between patients and volunteers. Further work is required to clarify this issue, since it could impact our understanding of the importance of protein binding in the PK of these drugs, in terms of volume of distribution, clearance and half-life.

We found lopinavir and ritonavir to bind to plasma proteins at 1.1% and 0.7% respectively. Small displacements to a highly bound drug by a potent displacer or fluctuations in the amount of plasma proteins could lead to large increases in unbound drug and have a profound effect *in vivo.*[283] For example, co-administration of an AAG displacer clindamycin in humans led to an increase in the intracellular concentration of imatinib, an oncogenic fusion protein highly bound to AAG. Within 5-10 in of bolus clindamycin administration to patients, the plasma concentrations increased from 2.6 – 4.7 fold. This was confirmed by *in vitro* experiments where imatinib intracellular concentrations reduced by 10-fold in the presence of AAG and this was reversed when incubated by clindamycin.[284]

Quinidine is highly bound to AAG[285] and sodium valproate is highly bound to albumin.[286] An increase in the unbound drug concentration of PIs can safely be attributed to displacement from AAG and albumin by quinidine and sodium valproate, respectively. Atazanavir,[282] lopinavir[261] and ritonavir[261] have been shown to bind to both AAG and albumin. However, in our study, AAG displacer quinidine failed to influence the proportion of unbound drug. A possible explanation is quinidine has a weaker affinity to AAG than atazanavir, lopinavir and ritonavir. An inhibitor with a stronger affinity for AAG could influence the binding of these PIs.

A combination of all AAG and albumin displacers resulted in higher displacement of all the PIs compared to the displacement by AAG and albumin displacers alone, which suggests that a compensatory mechanism was in operation. This observation was confirmed by darunavir displacement experiments in buffer containing AAG and albumin (**Figure. 4.7**). These experiments were performed to confirm that AAG and albumin are the plasma proteins responsible for the binding profile of the protease inhibitors, without involvement of any trace plasma proteins. Additionally, these experiments provided a confirmation that the displacers are displacing the protease inhibitors from the assigned plasma protein and not the other.

In the absence of albumin, binding to AAG was seen with 35.9 ± 0.1 % unbound. In the absence of AAG, albumin binding resulted in 14.6 ± 1.7% unbound darunavir and was similar to the unbound percentage of darunavir in plasma. Similar findings were reported by Mackichan and Zola where an AAG displacer caused 31% increase in the unbound carbamazepine in buffer containing AAG and only 12% in plasma. This difference was attributed to the presence of albumin in plasma available for binding.[287] This may represent an evolutionary mechanism to prevent high unbound

concentrations of substances that could result in toxicity, and may explain why protein binding displacement seldom causes significant clinical DDIs.[154] Although rare, these DDIs could have clinical implications when coupled with conditions where AAG and albumin levels in the body are altered. For example, Wu *et al*. demonstrated the decrease in total RO4929097, a γ-secretase inhibitor, in the presence of vismodegib in patients with breast cancer.[288]

A limitation of this work is that the data cannot be used directly to predict DDIs between the tested drugs in a clinical environment. Since the study was aimed to analyse the binding affinity of the PIs, the concentrations of the displacers – quinidine, sodium salicylate, ibuprofen, and sodium valproate – were much higher than the concentrations found *in vivo.* However, the data improve our understanding of the protein binding for PIs and this may be useful to support more accurate modelling and simulation in future work. Additionally, the pooled plasma used for this study was derived in 2 batches from healthy patients. The plasma protein concentration was not measured and was assumed to be the average found in healthy individuals. Changes in AAG and albumin have shown to differ in HIV-infected individuals compared to healthy individuals and may give rise to discrepancies in the findings.[212]

The displacement of protease inhibitors depends on the concentration and the equilibrium dissociation constant (K_D) of the displacers. Similar studies using multiple displacers at variable concentrations are needed to corroborate the findings and provide a better understanding of the displacement.

In conclusion, the protein binding percentage of darunavir and atazanavir were found to differ from values that have been stated previously within the literature. All the PIs were displaced by sodium valproate and none by sodium salicylate and ibuprofen.

This shows the tested PIs bind to the bilirubin site on albumin. The plasma protein binding profile of drugs to plasma proteins depends on multiple variables such as physiology, co-morbidities and binding affinity of host drug as well as co-administered drug. This warrants a case by case investigation on the effects of plasma protein binding of drugs on PK in relation to DDIs.

Chapter 5

Inhibitory Effects of Commonly Used Excipients on P-glycoprotein *in vitro*

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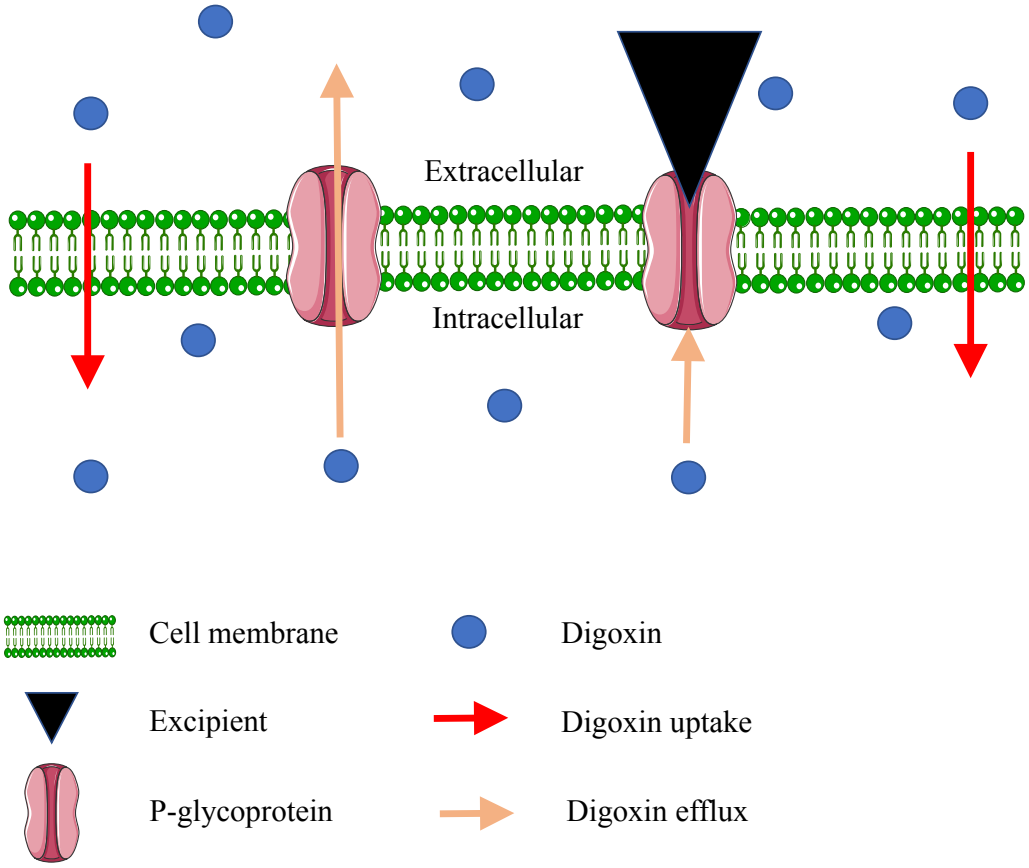

An abstract graphic showing the inhibition of efflux transporter P-gp by excipients. The efflux of P-gp substrate digoxin is inhibited.

5.1 Introduction

The International Pharmaceutical Excipients Council (IPEC) defines excipients as "substances other than the active pharmaceutical ingredient (API), which have been appropriately evaluated for safety and are intentionally included in a drug delivery system. They can aid in processing of the drug delivery system during its manufacture, protect, support or enhance stability, bioavailability and patient acceptability, assist in product identification, or enhance any other attribute of the overall safety, effectiveness or delivery of the drug during storage or use."[289] Excipients are used to bring about changes in the pharmacological activity of the drug by altering solubility, dissolution, permeability and bioavailability.[290,291]

Excipients were initially believed to be inert, with no biological activity of their own. However, several recent studies have demonstrated changes in the transporter-mediated absorption of substrates[292,293,294] and CYP enzyme-mediated metabolism *in vitro*.[295,296,297] The clinical impact of these biological effects has also been demonstrated. For example, poly(ethylene glycol) 400 was shown to significantly increase the bioavailability of ranitidine by inhibiting P-gp.[298] Interestingly, this inhibition was seen only in males and was hypothesised to be due to the lower activity and expression of P-gp in females compared to males.[299] Moreover, toxicity exhibited by some excipients has been a major concern for manufacturers.[300,301] For example, propylene glycol has been associated with central nervous system toxicity, cardiac arrhythmias, seizures, and lactic acidosis.[302] The need to study the potential pharmacological benefits and toxicological liabilities is therefore becoming increasingly apparent.

P-gp is an efflux transporter and belongs to the ABC family. This transporter is found in liver, kidneys, GI tract, placenta, and blood brain barrier in humans,[303] providing a protective mechanism against xenobiotics and toxins by restricting uptake and facilitating clearance via hepatocytes and renal tubules.[304] P-gp is also over-expressed in cancer cells leading to multidrug resistance.[305,306] A change in its activity through inhibition or induction can cause significant changes in the disposition and PK of substrate drugs which are the basis of DDIs. Although DDIs often lead to a negative impact on therapy, they can also be exploited in drug regimens to facilitate the absorption of drugs and increase their bioavailability.[307,308]

In previous studies, excipients such as Vit-E-PEG (TPGS) and Pluronic have been shown to inhibit P-gp activity in MDCK-MDR1 and Caco-2 cells respectively.[309,310] The clinical relevance of P-gp inhibition in the presence of excipients has been shown previously as Vit-E-PEG increased the oral absorption of paclitaxel and cyclosporine *in vivo*.[311,312] These changes in substrate bioavailability illustrate the importance of characterising the biological effects of excipients and their overall clinical impact.

In this study, the effect of 25 commonly-used pharmaceutical excipients on P-gp activity in MDCK-MDR1 cells was investigated. MDCK-MDR1 cells overexpress P-gp and their use to study P-gp circumvents the problems of complex cross-interactions between multiple transporters in non-transfected cell systems.[313] The MDCK-MDR1 cells were selected in accordance with the International Transporter Consortium for investigating the role of membrane transporters in drug development.[314] The findings of this study not only corroborate the previously reported effects of excipients on P-gp but also present novel effects of some excipients not previously reported.

5.2 Materials and Methods

5.2.1 Materials

Tritiated [3H]-digoxin (250 μCi/mmol) was purchased from Perkin Elmer (USA). Digoxin, verapamil, PVPP (Poly(vinylpolypyrrolidone)), magnesium stearate and Kollicoat were purchased from Sigma Aldrich (UK). Sisterna 16 (sucrose palmitate) was gifted from Sisterna (Netherlands). The remaining excipients were obtained from Sigma Aldrich (UK). MDCK-MDR1 cells were purchased from American Type Culture Collection (ATCC). Scintillation cocktail was obtained from Meridian Biotechnologies (UK) and the CellTiter-Glo® Luminescent Cell Viability Assay was purchased from Promega (UK).

5.2.2 Study Design

The toxicity of the drugs and excipients exhibited on MDCK-MDR1 cells were analysed. The cellular accumulation of digoxin in MDCK-MDR1 cells was first tested in the presence of 10 μM and 200 μM of each excipient. Excipient concentrations were chosen to span the range of excipient concentrations previously used for investigating activities *in vitro*. Molar concentrations were used to measure the effect of excipients on the accumulation of digoxin. Since PVPP is a cross-linked chain the determination of molecular weight was not possible. Hence, experiments for PVPP were performed by weight (μg/mL) concentrations. Excipients that showed a significant effect on accumulation of digoxin in MDCK-MDR1 at either of the tested concentrations were further studied for their concentration-dependent effect on P-gp. Logarithmic concentrations ranging from 0.33 - 1000 μM were used for Brij 58, Vit-

E-PEG, AOT, Tween80, Tween20 and Cremophor EL. The log concentrations used for Solutol HS 15 ranged from 1- 3333 μM to ensure the inclusion of higher concentrations necessary for accurate 50% inhibitory concentration (IC_{50}) determination. Due to high molecular weight of NaCMC, the maximum soluble concentration was at 250 μM, resulting in a log concentration range of 0.33- 250 μM.

Stocks of digoxin and verapamil were made in DMSO. The effect of the vehicle DMSO on the ATP assays and cellular accumulation assays were assessed to ensure lack of interference.

5.2.3 Excipients

A total of 25 excipients were chosen based on the previous use of these excipients by the investigators in the manufacture of solid drug nanoparticles.[315,316,317] During this manufacturing process these excipients are either used as polymers or as surfactants and these have been listed as follows:

Surfactants: Sodium deoxycholate (NaDC, MW = 414 g/mol), sodium caprylate (NaCap, MW = 166 g/mol), D-α-tocopherol poly- (ethylene glycol) succinate (Vit-E-PEG, MW = 1000 g/mol), sucrose stearate (Sisterna 11, MW = 608 g/mol), sucrose palmitate (Sisterna 16, MW = 580 g/mol), sodium 1,4-bis (2- ethylhexoxy)-1,4-dioxobutane-2-sulfonate (AOT, MW= 444 g/ mol), poly(ethylene oxide)$_{35}$ modified castor oil (Cremophor EL, MW = 2500 g/mol), polyethylene glycol$_{15}$-hydroxystearate (Solutol HS 15, MW = 345 g/mol), poly (ethylene oxide)$_{20}$ sorbitan monolaurate (Tween 20, MW = 1230 g/mol), poly (ethylene oxide)$_{20}$ sorbitan monooleate (Tween 80, MW = 1300 g/mol), poly (ethylene glycol) hexadecyl ether (Brij 58, MW = 1124

g/mol), alkyl (C_{12-16}) dimethylbenzylammonium chloride (Hyamine, MW = 448 g/mol), cetyltrimethyl- ammonium bromide (CTAB, MW = 364 g/mol), magnesium stearate (MW= 591.27 g/mol).

Polymers: Hydroxypropyl cellulose (HPC, MW = 80000 g/mol), hydroxypropylmethyl cellulose (HPMC, MW = 10000 g/mol), hydrolysed gelatin (HG, MW = 1980 g/mol), sodium carboxymethylcellulose (NaCMC, MW = 90000 g/mol), poly(ethylene glycol) (PEG, MW = 1000 g/mol), poly (ethylene oxide)$_{80}$-block-poly (propylene oxide)$_{27}$-block-poly (ethylene oxide)$_{80}$ (Pluronic F68, MW = 8400 g/mol), poly (ethylene oxide)$_{101}$-block- poly (propylene oxide)$_{56}$-block-poly (ethylene oxide)$_{101}$ (Pluronic F127, MW = 12600 g/mol), poly (vinyl alcohol)-graft-poly (ethylene glycol) copolymer (Kollicoat, MW = 45000 g/mol), poly (vinyl alcohol) (80% hydrolysed PVA, MW = 9500 g/mol), poly (vinyl pyrrolidone) (PVP K30, MW = 40000 g/ mol), Poly (vinylpolypyrrolidone) (PVPP).

Stocks of excipients were freshly prepared in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS).

5.2.4 Culture of MDCK-MDR1 cells

MDCK-MDR1 cells were grown in DMEM supplemented with 10% FBS. The cells were seeded in a T175 flask and incubated in a humidified incubator (37°C, 5% CO_2). Media was changed every 48 hours. They were sub-cultured by standard trypsinisation and centrifugation and passaged at 80% confluency. Cells were discarded beyond passage 20. Differences in the experimentation due to variation of

passages were monitored by ensuring lack of significant changes in the positive and negative controls in the experiments.

5.2.5 Cell Counting and Viability

The cell numbers and viability of Caco-2, MDCK and MDCK-MDR1 cells were calculated using the Chemometec NucleoCounter® NC-100TM (Chemometec, Denmark). A volume of 150 µl of cell suspension was added to a fresh Eppendorf tube for total dead cells count, 50 µl of reagent A and B were added to 50 µl of cell suspension in a separate Eppendorf tube for total cell count. A cell viability of > 95% was considered acceptable for the experiments.

Cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = 100 - \left(\frac{\text{Dead cells count}}{\text{Total cells count (Reagent A + B) x 3 (dilution factor)}}\right)$$

5.2.6 Cytotoxicity Assay

The cytotoxicity of excipients and drugs used in the experiment were assessed by measuring the cell viability of MDCK-MDR1 after exposure, using the CellTiter-Glo® Luminescent Cell Viability Assay.[317] This assay measures the number of metabolically active cells by quantifying the amount of ATP present.[318] The assay was performed in quadruplicates. MDCK-MDR1 cells were seeded in a 96-well plate at 20,000 cells/well in 100 µl of DMEM with 10% FBS overnight to allow for adherence to the culture plate. A row of cells without treatment with drugs or

excipients was included as negative controls and wells containing media without cells were included to normalise for background luminescence. After 24 hours, media was removed and the cells were exposed to drugs and excipients at concentrations ranging from 0.97 - 1000 μM (0.97 – 1000 μg/mL for PVPP) (diluted in media) and incubated in a humidified incubator (37°C, 5% CO_2) for 1 hour to ensure complete P-gp inhibition and equilibrium between the intracellular and extracellular digoxin. Post-incubation, the plate was equilibrated to room RT for 30 minutes. A volume of 100 μl of CellTiter-Glo® Reagent was added to each well and mixed on an orbital shaker for 2 minutes to induce lysis. The plate was incubated at RT for 10 minutes to ensure stabilisation of the luminescent signal. The luminescence was then measured using a GENios Tecan microplate reader (Germany). The background luminescence was subtracted and the viability was calculated as a percentage of untreated cells assuming 100% viability.

5.2.7 Determination of Cellular Accumulation of Digoxin

MDCK-MDR1 cells were seeded in 4 wells of a 6-well plates at 1 x 10^6 cells/mL and incubated for 24 hours in a humidified incubator (37°C, 5% CO_2). Digoxin (10 μM) was used as a P-gp substrate and verapamil (10 μM), a P-gp inhibitor was used as positive control in accordance to the FDA guidelines 2006.[319,320] Aliquots were prepared with media containing digoxin alone or in the presence of verapamil or excipients. [3H]-digoxin at 0.1 μCi/mL was added to the aliquots along with non-radiolabelled digoxin. The cells were then exposed to these aliquots and incubated for 1 hour in a humidified incubator (37°C, 5% CO_2). After incubation, 100 μl of the supernatant fraction was taken and transferred to a scintillation vial to determine the

extracellular substrate concentration. The cells were then washed 3 times with cold HBSS to remove the remaining culture media. Cells were removed from the culture plate by trypsinisation with 1mL of trypsin-EDTA per well and incubation for 10 minutes in a humidified incubator (37°C, 5% CO_2). After incubation, the cells were agitated for detachment and transferred to scintillation vials for measurement of intracellular substrate concentrations. A volume of 4 mL of scintillation cocktail was added to the vials and the radioactivity count was measured by a Packard Tri-Carb 3100 TA Liquid Scintillation Counter (Perkin-Elmer, Cambridge, UK).

5.2.8 Data Analysis

Cytotoxicity of drugs were calculated as percentage of cells alive after exposure to varying concentrations of drugs and excipients compared to cells in the control group. The background luminescence was subtracted from the luminescence given by cells exposed and in the control group. Cell viability was then calculated using the formula:

$$\text{Cell Viability } \% = \frac{\text{Luminiscence of cells after exposure}}{\text{Luminiscence of cells in control}} \times 100$$

Cellular accumulation ratio for digoxin was calculated using the following formula (where DPM = disintegrations per minute):

$$CAR = \frac{(intracellular\ DPM/total\ cell\ volume)}{(extracellular\ DPM/extracellular\ volume)}$$

Cellular volumes were determined using the Scepter™ cell counter 2.0 (Merck Millipore, Billerica USA). Cell volumes were taken from a mean of 3 replicates, MDCK-MDR1 volume 3.7 pl.

Normality of the data was tested using a Shapiro-Wilk test after which statistical significance was assessed using an unpaired t-test using SPSS 22.0. The difference in cellular accumulation of digoxin alone (0%) and in the presence of verapamil (100%) was used to calculate the percentage increase in accumulation of digoxin due to excipients. IC_{50} for the excipients that were tested for concentration-dependent inhibition of P-gp was calculated using Graph-pad Prism (Version 6).

5.3 Results

5.3.1 Cytotoxicity of Excipients on MDCK-MDR1

Digoxin, verapamil, Vit-E-PEG, HPC, Solutol HS 15, Cremophor EL, PVA, PVP K30, PEG, Pluronic F68, Pluronic F127, HPMC, HG, NaCap, Sisterna 11, Kollicoat, Tween 20, Tween 80, PVPP and magnesium stearate did not exhibit any cytotoxicity at the tested concentrations (>80% viability). Cytotoxicity was observed with NaDC, AOT, Brij 58, Hyamine, CTAB, NaCMC and Sisterna 16 (**Figure. 5.1**).

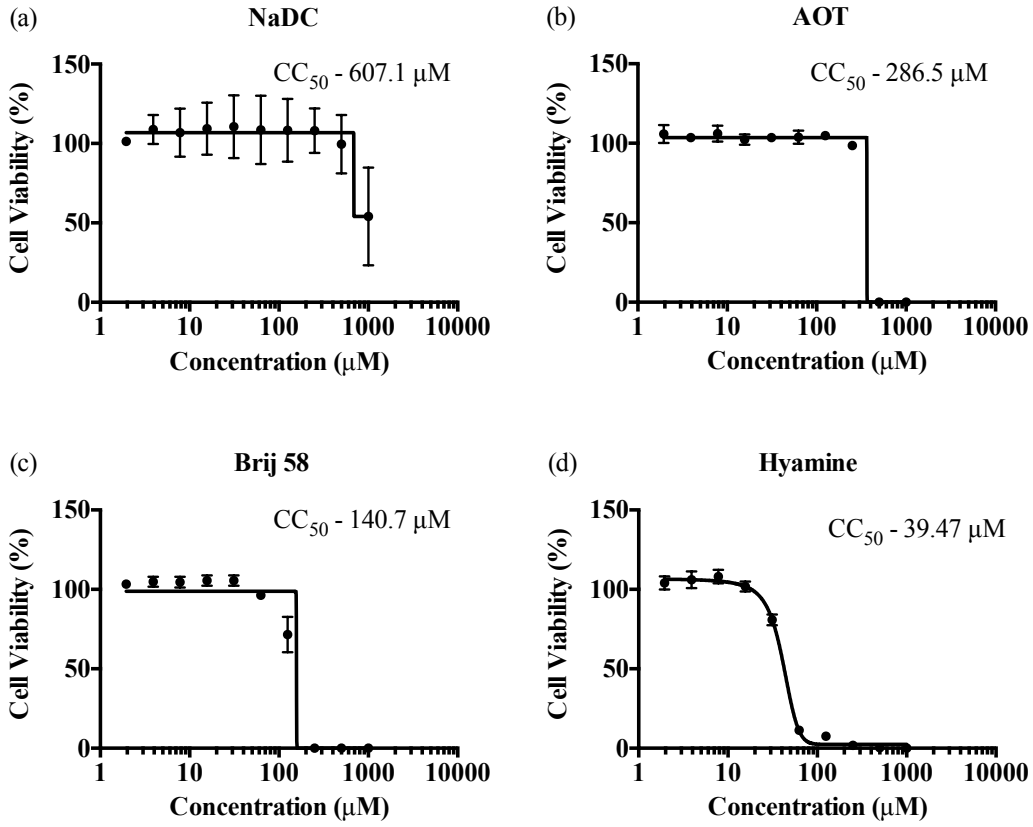

Figure 5.1. Toxicity exhibited by (a) NaDC, (b) AOT, (c) Brij 58, (d) Hyamine on MDCK-MDR1 cells measured by CellTiter-Glo® assay. Data are presented as mean ± standard deviation. n = 4.

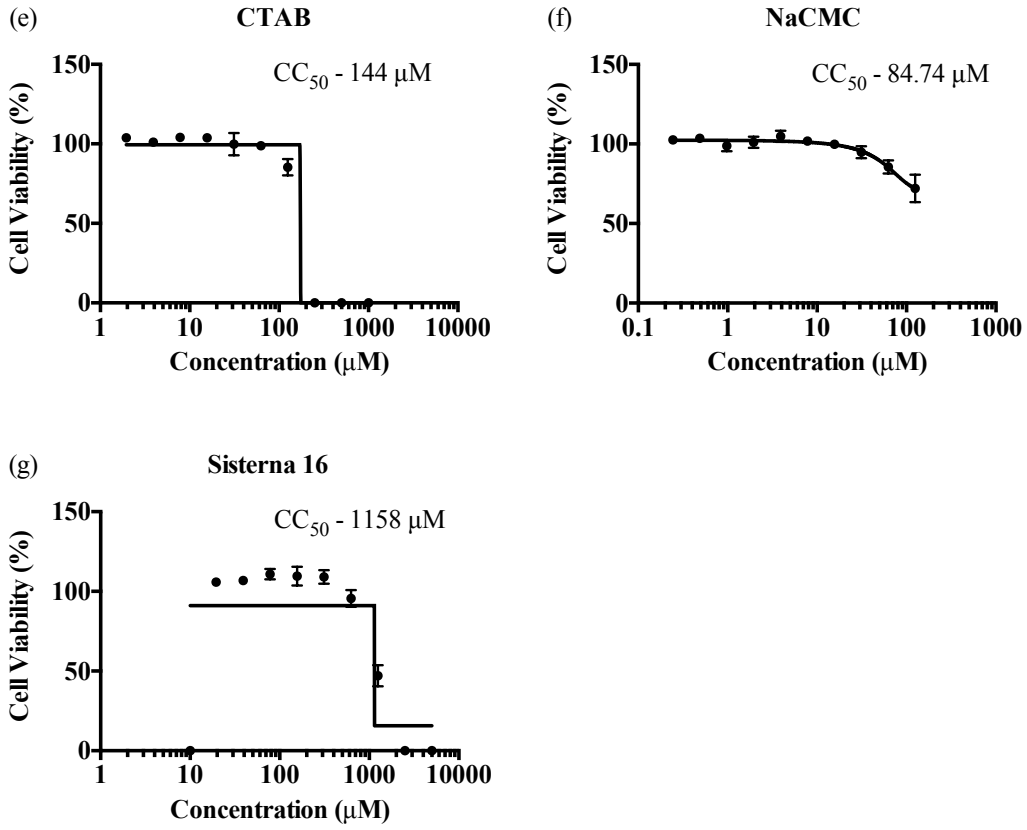

Figure 5.1. continued. Toxicity exhibited by (e) CTAB, (f) NaCMC, (g) Sisterna 16 on MDCK-MDR1 cells measured by CellTiter-Glo® assay. Data are presented as mean ± standard deviation. n = 4.

5.3.2 Change in Cellular Accumulation of Digoxin in MDCK-MDR1 due to Excipients

At 10 μM a significant increase in the cellular accumulation of digoxin (**Figure. 5.2a**) was seen in the presence of Vit-E-PEG (290.8 ± 60.9 % increase, p = 0.002), Tween 80 (110.6 ± 23.3% increase, p = 0.001), CTAB (22.2 ± 8.3% increase, p = 0.021), Cremophor EL (37.2 ± 15.6% increase, p = 0.01), Solutol HS 15 (27.6 ± 10% increase, p = 0.006) and Brij 58 (38.1 ± 8.9% increase, p = 0.001).

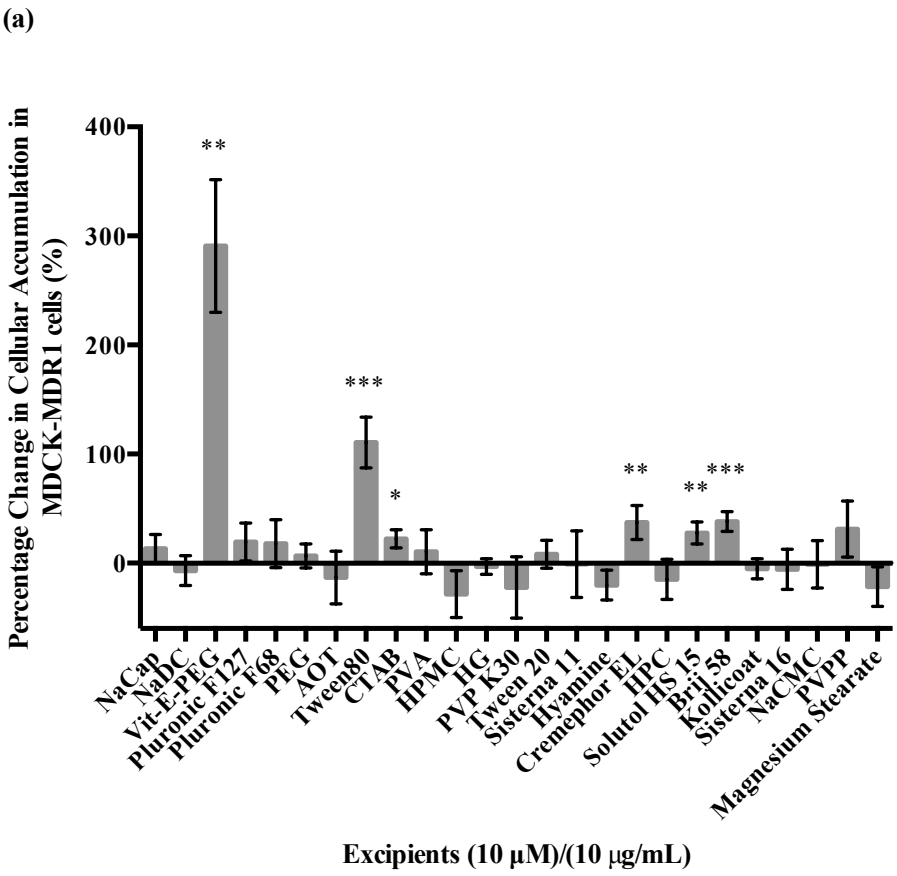

Figure 5.2 (a). Percentage change in the accumulation of digoxin caused by excipients at 10 μM (or 10 μg/mL for PVPP) compared to the change in accumulation of digoxin due to verapamil in MDCK-MDR1 cells. Data are presented as mean ± standard deviation. n = 4. Statistical significance was tested using an unpaired t-test. P Value: *, < 0.05; **, < 0.01; ***, < 0.001; ****, ≤ 0.0001.

At 200 μM, Vit-E-PEG (306.8 ± 34% increase, $p < 0.0001$), AOT (206.4% ± 34.3 increase, $p < 0.0001$), Tween 80 (314.7 ± 48.5% increase, $p < 0.0001$), CTAB (511.9% ± 135.4% increase, $p = 0.004$), Tween 20 (485.5 ± 35.3% increase, $p < 0.0001$), Cremophor EL (215.4 ± 35.8% increase, $p < 0.0001$), Solutol HS 15(373.6 ± 33.9% increase, $p < 0.0001$), Brij 58 (619.3 ± 55.7% increase, $p < 0.0001$) and NaCMC (182 ± 65.3% increase, $p = 0.006$) significantly increased the cellular accumulation of digoxin in MDCK-MDR1 cells (**Figure 5.2b**).

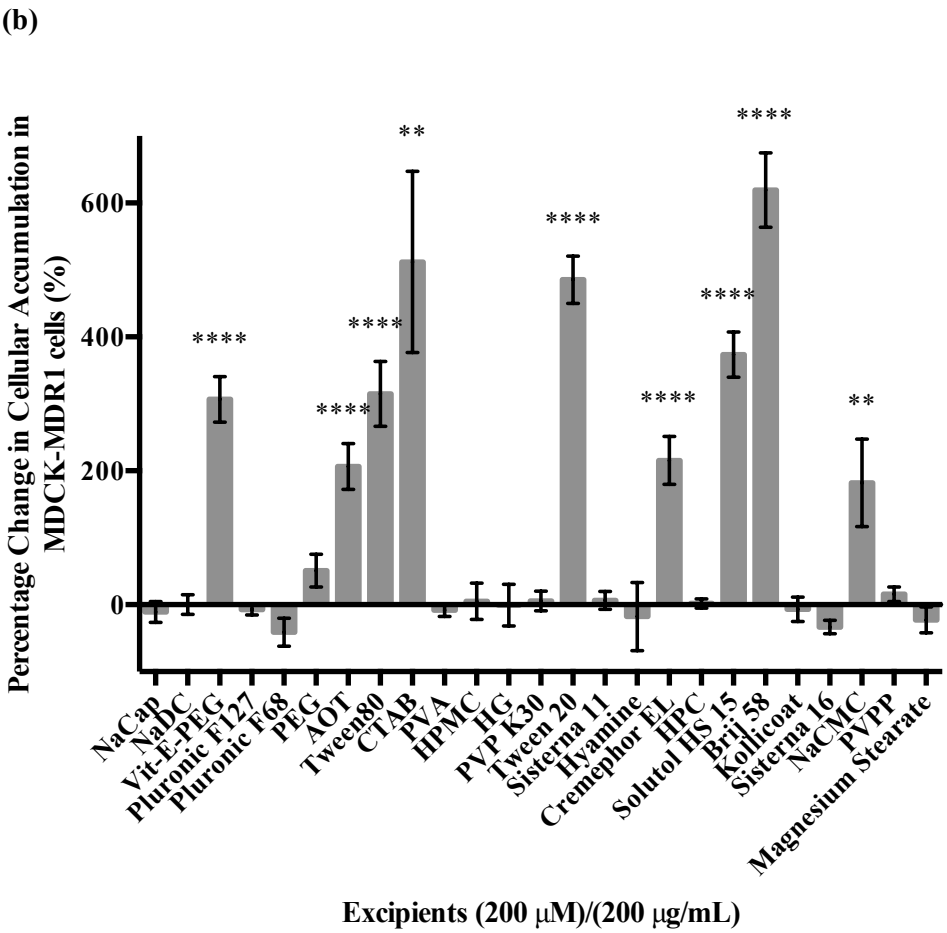

Figure 5.2 (b). Percentage change in the accumulation of digoxin caused by excipients at 200 μM (or 200 μg/mL for PVPP) compared to the change in accumulation of digoxin due to verapamil in MDCK-MDR1 cells. Data are presented as mean ± standard deviation. n = 4. Statistical significance was tested using an unpaired t-test. P Value: *, < 0.05; **, < 0.01; ***, < 0.001; ****, ≤ 0.0001.

A concentration-dependent increase in the intracellular digoxin was seen (**Figure. 5.3**) with Vit-E-PEG (IC50 = 12.48 μM), AOT (IC50 = 192.5 μM), Tween 80 (IC50 = 45.29 μM), CTAB (IC50 = 96.67 μM), Tween 20 (IC50 = 74.15 μM), Cremophor EL (IC50 = 11.92 μM), Solutol HS 15 (IC50 = 179.8 μM), Brij 58 (IC50 = 25.22 μM) and NaCMC (IC50 = 46.69 μM).

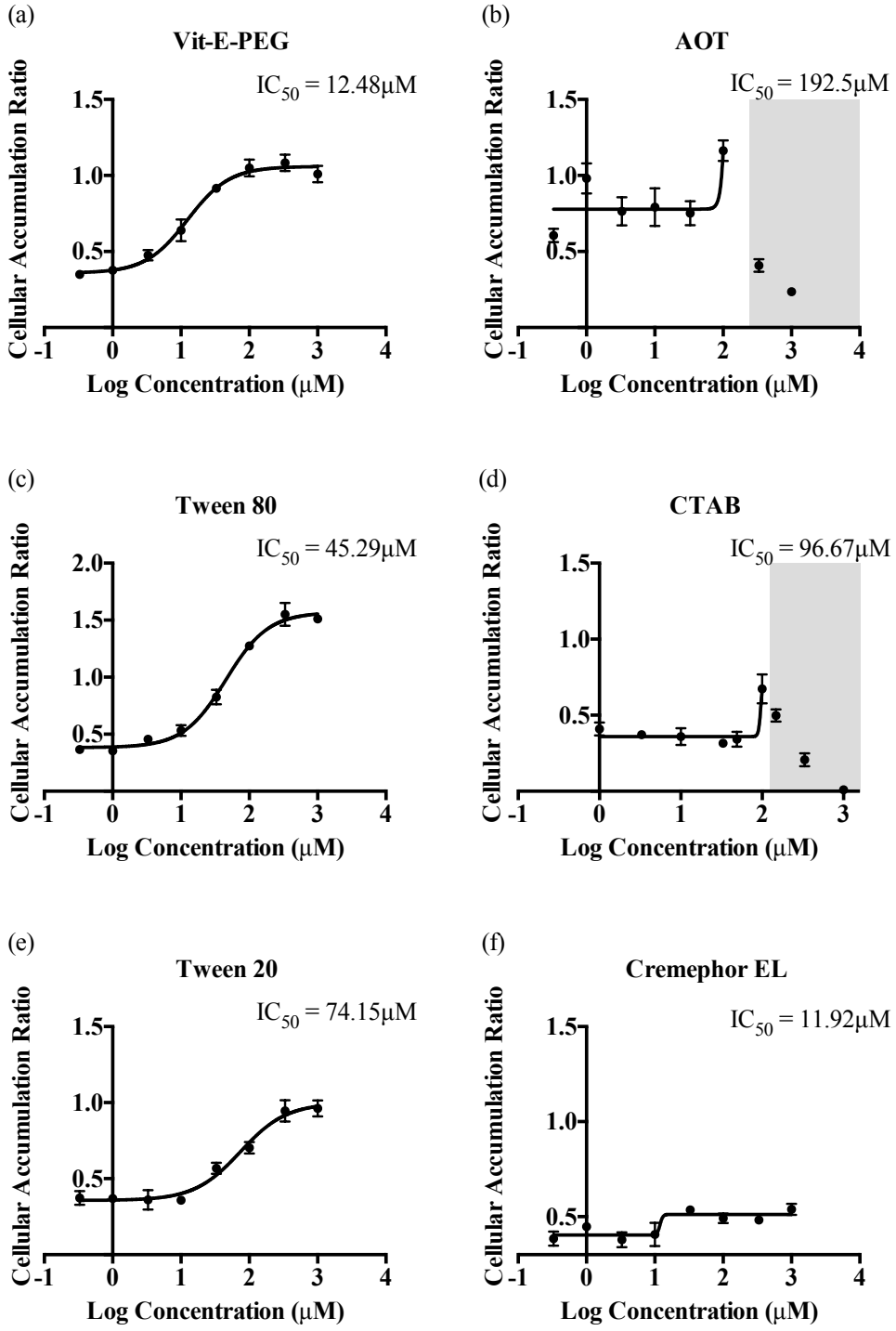

Figure 5.3. Concentration-dependent increase in digoxin accumulation in MDCK-MDR1 cells by (a) Vit-E-PEG, (b) AOT, (c) Tween 80, (d) CTAB, (e) Tween 20, (f) Cremophor EL. Data are presented as mean ± standard deviation. n = 4. Shaded area represents the concentrations at which excipients exhibited cytotoxicity towards MDCK-MDR1 cells.

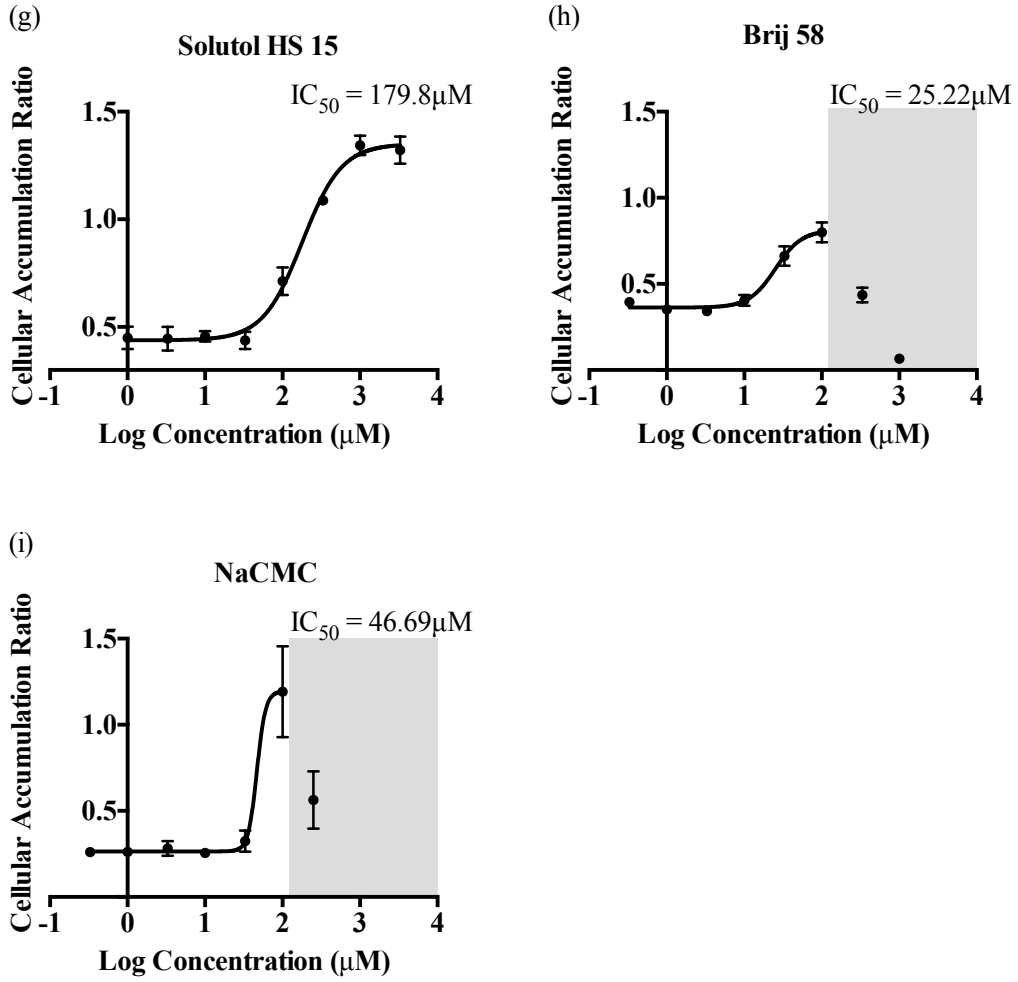

Figure 5.3 continued. Concentration-dependent increase in digoxin accumulation in MDCK-MDR1 cells by (g) Solutol HS 15, (h) Brij 58, and (i) NaCMC. Data are presented as mean ± standard deviation. n = 4. Shaded area represents the concentrations at which excipients exhibited cytotoxicity towards MDCK-MDR1 cells.

5.4 Discussion

MDCK-MDR1 cells overexpress P-gp and are a useful cellular model for identifying substrates and inhibitors of P-gp. The effect of other transporters that would potentially mask any interactions with P-gp transport in a non-transfected cell system is comparatively low. Therefore, an increase in the cellular accumulation of digoxin can be safely attributed to the inhibition of P-gp activity.

At 10 μM, NaCAP, AOT, HPMC, PVP K30, Hyamine, HPC and magnesium stearate led to a decrease in digoxin accumulation. Similarly, at 200 μM, NaCAP, Pluronic F127, Pluronic F68, PVA, Hyamine, Kollicoat, Sisterna 16 and magnesium stearate decreased digoxin accumulation. These changes however were not statistically significant and might be a result of variability seen in experimentation due to the use of individual wells.

In this study, the concentration-dependent inhibition of the efflux transporter P-gp by commonly-used pharmaceutical excipients was clearly demonstrated by the following order of effects: Cremophor EL > Vit-E-PEG > Brij 58 > Tween 80 > NaCMC > Tween 20 > CTAB > Solutol HS 15 > AOT.

Surfactants are extensively used in formulations as emulsifying agents to dissolve drugs with poor solubility and enhance their bioavailability.[321] The have been shown to affect the P-gp's ATPase activity through a concentration dependant alteration in the fluidity of the lipid bilayers of cell surface membranes.[322] When exposed to MDCK-MDR1 cells, this modulation in fluidity can inhibit the efflux of P-gp substrates like digoxin and result in an increase in their cellular accumulation. An alternative mechanism has been proposed where excipients inhibit P-gp by inhibiting protein kinase C, which plays a role in the functioning of the P-gp transporter.[323]

Vit-E-PEG is an esterified Vitamin E derivative, widely used as a solubilising and emulsifying agent.[324] The *in vivo* effects of Vit-E-PEG on P-gp substrates have been demonstrated in previous studies. For example, TPGS was seen to increase the AUC and reduce oral clearance of cyclosporine in healthy patients.[312] Since the inclusion of Vit-E-PEG in the list of GRAS (Generally Recognised As Safe) substances by FDA, there has been an attempt to include it in formulations to increase the bioavailability of drugs by inhibiting P-gp.[325,326] In our study, Vit-E-PEG had a low IC_{50} (12.48 μM) for inhibition of P-gp suggesting higher possibilities of interactions even at lower concentrations. Interestingly, the results published by Collnot *et al*. suggest that Vit-E-PEG inhibits P-gp by either blocking the binding of a substrate to the transporter or by allosteric modification of P-gp, rather than changing membrane fluidity of cells.[327] These discrepancies indicate the need for case-by-case investigations of surfactants to elucidate their mechanism of P-gp inhibition.

Cremophor EL, a modified castor oil, is widely used as emulsifying agents to stabilize microemulsions.[328] Out of all the excipients tested in this study, Cremophor EL was the most potent inhibitor (IC_{50} = 11.92 μM) of P-gp. The *in vitro* and *in vivo* inhibition of P-gp by Cremophor EL has been well-characterised in the past and has been suggested for use in formulations.[329,328] For example Taxol is routinely used for chemotherapy and contains 6 mg paclitaxel with 527 mg of purified Cremophor EL and 49.7% (v/v) dehydrated alcohol. However, toxicity and hypersensitivity reactions have made the formulation unpopular, driving manufacturers to look for alternatives to Cremophor EL.[330,331]

Solutol HS 15 has been studied for its P-gp inhibitory effects *in vitro* and *in vivo* and is extensively used to improve bioavailability of drugs.[310,332] Micelles made from

Solutol HS 15 have been shown to increase the bioavailability and the antitumor efficacy of paclitaxel due to its P-gp inhibitory activity.[333] In our study, Solutol HS 15 significantly inhibited P-gp with an IC_{50} of 179.8 μM.

Tween 20 and Tween 80 are polysorbates (fatty acid esters of sorbitol) that inhibit P-gp and their effects on the PK of drugs that are substrates of P-gp have been demonstrated *in vitro* and *in vivo*.[310,334,335,336] These findings are corroborated by our results where Tween 20 and Tween 80 inhibited P-gp with IC_{50}'s of 74.15 μM and 45.29 μM, respectively.

Brij molecules such as Brij 30[337] and Brij 35[338] have been shown to inhibit efflux transporters resulting in enhanced absorption of drugs. Inhibition of P-gp along with depletion of ATP due to Brij 78 has also been established.[339] However, there is currently limited data on the effects of Brij 58 on P-gp. Tang *et al*. have reported increased efficacy of paclitaxel when combined with Brij 58 which led to an anti-proliferation effect and inhibition of cell growth in H460/taxR cells that overexpress P-gp.[340] To our knowledge, no other studies have measured the effect of Brij 58 on P-gp. In our study, we have shown Brij 58 to be a strong inhibitor of P-gp with an IC_{50} of 25.22 μM.

Previously, AOT had been shown to inhibit P-gp in bovine brain microvessel endothelial cells that overexpress P-gp. The use of AOT-alginate nanoparticles has resulted in increased cellular delivery of drugs resulting in enhanced efficacy.[341,342] For example, methylene blue AOT-alginate particles showed enhanced photodynamic activity in MDC-7 cells.[343] Similarly, our results show inhibition of P-gp by AOT with an IC_{50} of 192.5 μM.

Nanoparticles of CTAB have shown to increase the intracellular concentrations of P-gp substrates and when combined with a known P-gp inhibitor, which led to complete reversal of multidrug resistance in cancer cells.[344,345] Singh *et al*. showed an increase in intracellular rhodamine-123 (R-123) by CTAB nanoparticles in glioblastoma and gliosarcoma cells and this effect was attributed to evasion of efflux transporters.[332] Similarly, our results show that CTAB inhibits P-gp with an IC_{50} of 96.67 μM.

The surfactants: NaDC, NaCap, Sisterna 11, Sisterna 16, Hyamine and magnesium stearate, did not have any significant effect on the digoxin cellular accumulation. To our knowledge, there are no studies that demonstrate the effects of these excipients on P-gp.

Polymers comprise of a varied group of substances that are synthesised as well as derived from natural products. Depending on their molecular weight, they have a wide range of physiochemical properties[346] and are used in formulating drugs for all routes of administrations.[347] P-gp inhibition by polymers has been demonstrated in the past. Polymers such as Pluronic P85 and PEG 400 show emulsifying and solubilising properties and are used as surfactants in formulations.[348,349] They have shown to inhibit P-gp by modulating the membrane fluidity, similar to the surfactants described above.[350] Furthermore, thiolated polymers have also demonstrated P-gp inhibition by entering the channels of P-gp transporter and forming disulphide bonds with the cysteine subunits, rendering P-gp inactive.[351]

Sodium carboxymethylcellulose (NaCMC/CMC) is a binding agent that is not only used in pharmaceutical formulations but also in everyday foods. It is used for its viscosity increasing properties and to suspend powders for oral and parenteral administration.[352] Additionally, it is used to stabilise emulsions.[353] Docetaxel is a

chemotherapy drug used in the treatment of breast, prostate and lung cancers. One of the major hurdles of this treatment is tumour resistance, which is attributed to upregulation of P-gp causing efflux of chemotherapy drugs from target tumour cells.[354] Using NaCMC and PEG, a polymer conjugate Cellax was developed. Cellax was successful not only in improving the efficacy of docetaxel, but also circumventing resistance developed by the tumour cells in cell and mouse models.[355,356,357] To our knowledge, the inhibitory effect of NaCMC on the accumulation of a P-gp substrate in P-gp overexpressing cells has been demonstrated for the first time in this study. This P-gp inhibiting characteristic could be a factor contributing to the success of Cellax in treating cancer.

The polymers tested (HPC, HPMC, HG, PEG 1000, Kollicoat, PVA, PVP, Pluronic F68, Pluronic F127 and PVPP) in this study did not show any significant P-gp inhibition. To our knowledge, there are no published studies that state otherwise. Although, PEG 300 and PEG 400 have been shown to have an inhibitory effect on P-gp, our results did not show any inhibition by PEG 1000.[358,359] This was in agreement with a study by Collnot *et al*. where PEG 1000 did not have any effect on the apparent permeability of R-123 in Caco-2 monolayers.[324] HPMC has been reported to reduce *MDR1* expression in LS174T cells.[296] However, no other studies have shown changes in P-gp substrate caused by HPMC.

Changes in the PK of P-gp substrates were seen in the presence of Pluronic F68 and F127 and was attributed to the inhibition of P-gp. For example, F68 increased the $AUC_{0\text{-}t}$ in rats and increased the permeability of celiprolol in Caco-2 cell monolayers.[360,173] Pluronic F127 was also seen to increase intracellular concentrations of R-123 in Caco-2 cells in a dose-dependent manner.[361] However, *in vitro* studies carried out to assess the effect of Pluronic F68 and Pluronic F127 on MDCK-MR1

cells showed no effect on the transport of a P-gp substrate.[362,363] Similarly, in our study, Pluronic F68 and Pluronic F127 did not affect the cellular accumulation of digoxin in MDCK-MDR1 cells. It appears that Pluronic F68 and F127 showed inhibition of P-gp in Caco-2 cells, but not in P-gp overexpressing MDCK-MDR1 cells. Further investigations are needed to pin-point the exact cause of this discrepancy.

P-gp in the gastrointestinal tract plays a major role in the bioavailability of orally administered drugs.[308] Additionally, P-gp is over-expressed in cancer cells and is responsible for MDR-related drug resistance.[305] The IC_{50} calculated in our study is an important measure to compare the P-gp inhibiting potency of different excipients. The IC_{50} calculated cannot be linked to in vivo concentrations since the concentration of excipients cannot be measured in the gut after administration. However, knowledge about the P-gp inhibiting properties of excipients will enable manufacturers to choose appropriate excipients to either exploit the P-gp inhibition for increasing the drug bioavailability or ovoid DDIs.

There are a few limitations in our study. There is the potential for binding by the excipients to the proteins contained within the culture medium; this could render a significant amount of the excipient unavailable for P-gp inhibition. Moreover, our study was carried out in one lot of MDCK-MDR1 cell lines and the results could vary with different lots of MDCK-MDR1 or different types of cells overexpressing P-gp. Similar experiments are required to establish reproducibility using different lots of cells are needed to corroborate our findings and strengthen meaningful extrapolations.

ATP assay was chosen as it is simple, cost-effective and sensitive compared to other assays. However, a limitation while using this assay is that the ATP assay used to

assess the toxicity of excipients can be affected by the presence of surfactants and detergents since they modulate the ATPase in cells.[322] Therefore, the toxicity of the excipients needs to be confirmed using additional methods.

In conclusion, most surfactants (Vit-E-PEG, AOT, Tween 80, CTAB, Tween 20, Cremophor EL, Solutol HS 15 and Brij 58) and the polymer NaCMC inhibited P-gp in MDCK-MDR1; demonstrated by the significant increase in intra-cellular digoxin. This disproves the inertness of excipients and ascertain the possibility of P-gp mediated DDIs due to excipients. Our findings, corroborated with published literature, suggest that excipients with surfactant properties have a greater propensity to inhibit P-gp. The biological effects of excipients on other transporters and drug metabolising enzymes need to be determined to understand the implications of APIs on the PK of drugs. This will help the manufacturers to choose the adequate excipient in formulations to bring about favourable drug pharmacokinetics.

Chapter 6

Effect of P-glycoprotein, MRP and BCRP Inhibitors on Transport of Linezolid: Implications for Drug-Drug Interactions and Resistance

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6.4 Discussion

6.1 Introduction

In this era of antibiotic resistance, efficient management to deliver quantity and quality of life is increasingly challenging.[364] MDR is eroding available pharmacological strategies to effectively control infections.[365] In 2016, the WHO estimated that 3.9% (95% CI 2.7-5.1%) of previously untreated and 21% (95% CI 15 - 28%) of previously treated TB cases worldwide were MDR-TB.[19] The demand for newer antibiotics with favourable pharmacokinetic and pharmacodynamic characteristics to replenish the exhausted inventory of drugs for the treatment of infections is evident.[366]

TB is now the most common OI amongst patients infected with HIV due to which ART is often co-administered with anti-tubercular treatment (ATT).[367] Both ART and ATT comprise multi-drug combinations, resulting in higher potential for DDIs. Drugs used as part of ART and ATT exhibit toxicities and when co-administered could add to the severity of adverse events. This could worsen the patients' condition and lead to treatment discontinuation, increasing the risk of loss MDR.[368] Moreover, patients infected with HIV have been shown to have a higher rate of MDR-TB compared to patients without HIV, which is partly attributed to DDIs and treatment discontinuation.[22] This highlights the need to characterise the metabolic profile of the drugs used in ART and ATT.

Linezolid, an oxazolidinone, acts on Gram-positive bacteria by inhibiting formation of the 70S initiation complex by binding to the 23S ribosomal RNA (rRNA) of the bacterial 50S ribosomal unit.[369] Since its approval by FDA in 2000, linezolid has been adopted for treatment of a wide range infections caused by organisms such as *Enterococcus faecalis, Staphylococcus aureus, Chlamydia pneumoniae,*

Haemophilus influenza, and MDR-TB.[174,370] The lack of cross resistance with other antibiotics makes linezolid an extremely favourable option.[371] Although linezolid has been used considerably for several pathogens, incidences of resistance have remained low. The LEADER surveillance program that analysed linezolid activity from 2011 to 2015 showed a modest <1% resistance to linezolid.[372]

Notwithstanding the advantages, linezolid has a low therapeutic index that makes treatment vulnerable to DDIs. A suboptimal exposure of the drug could result in resistance and treatment discontinuation.[373] An emergence of linezolid-resistant enterococci due to mutations in the 23S rRNA has been observed and has been correlated to prior use of linezolid.[374] Additionally, DDIs could lead to higher plasma levels of linezolid, resulting in adverse events and treatment discontinuation.[375] Treatment with linezolid is often associated with adverse events such as neuropathy, thrombocytopenia, anaemia and hyperlactatemia via a mitochondrial effect.[376] Careful monitoring of these adverse events, especially in children is therefore recommended.[377]

ABC and SLC transporters consist of more than 400 types of transmembrane proteins responsible for drug transport and DDIs.[378] Linezolid is not metabolised by CYP enzymes and is cleared via renal (35%) and non-renal mechanisms.[379] Linezolid is suspected to be a P-gp substrate, but no data currently exist to show substrate affinity for P-gp or other ABC transporters that mediate DDIs.[380] More drug transporter data are therefore needed to inform DDI prediction and management. Accordingly, the purpose of this study was to investigate substrate recognition by P-gp, BCRP and MRP transporters. Transporter substrate properties of linezolid were assessed across Caco-2 monolayers with or without P-gp, MRP and the BCRP inhibitors using verapamil[381], MK-571[382] and fumitremorgin C (FUMC),[383] respectively.

6.2 Methods

6.2.1 Materials

Linezolid was purchased from Stratech Scientific Ltd (UK). [3H]-Digoxin (250 μCi/mmol) and [14C]-Mannitol (60 μCi/mmol) was purchased from Perkin Elmer (UK). MDCK, MDCK-MDR1 and Caco-2 cells were purchsed from ATCC (American Type Culture Collection). Transwell plates were purchased from Corning technologies (USA). The CellTiter-Glo® Luminescent Cell Viability Assay was purchased from Promega (UK). The scintillation cocktail fluid used was obtained from Meridian Biotechnologies (UK). All other reagents were obtained from Sigma Aldrich (UK).

6.2.2 Study Design

Toxicity of drugs used in all the experiments were analysed. The substrate properties of linezolid were investigated by analysing the effect of transporter inhibitors on the transport of linezolid across Caco-2 cells in trans-well plates. Verapamil, FUMC and MK-571 were used as inhibitors for P-gp, BCRP and MRP transporters, respectively. To further corroborate the effect of verapamil, the accumulation of linezolid in MDCK and MDCK-MDR1 cells was measured in the presence and absence of verapamil.

6.2.3 Cell Culture and Maintenance

Cells were maintained in DMEM supplemented with 15% sterile filtered FBS for Caco-2 cells and 10% of sterile filtered FBS for MDCK and MDCK-MDR1 cells. The cells were seeded in a T175 flask and incubated in a humidified incubator (37°C, 5% CO_2). The media was changed every 48 hours. Cells were sub-cultured by standard trypsinisation and centrifugation method when they reached approximately 80% confluency.

6.2.4 Cell Counting and Viability

The cell numbers and viability of Caco-2, MDCK and MDCK-MDR1 cells were calculated using the Chemometec NucleoCounter® NC-100TM (Chemometec, Denmark). A volume of 150 µl of cell suspension was added to a fresh Eppendorf tube for total dead cells count, while 50 µl of reagent A and B were added to 50 µl of cell suspension in a separate Eppendorf tube for total cell count. A viability of > 95% was considered ideal for the experiments. Cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = 100 - \left(\frac{\text{Dead cells count}}{\text{Total cells count (Reagent A + B) x 3 (dilution factor)}}\right)$$

6.2.5 Cytotoxicity Assay

Cytotoxicity was assessed in Caco-2, MDCK and MDCK-MDR1 cells using the CellTiter-Glo® Luminescent Cell Viability Assay as previously described.[317] This assay measures the number of metabolically active cells by quantifying the amount of ATP present.[318] The assay was performed in quadruplicates. A total of 2 x 10^4 cells of Caco-2, MDCK and MDCK-MDR1 cells in 100 µl of media were plated in a flat 96-well plate and kept in a humidified incubator (37°C, 5% CO_2) to allow the cells to adhere. After 24 hours, the media from the plates were removed and new media containing serial dilutions of linezolid (1.95 µg/mL - 1000 µg/mL), digoxin (0.15 µg/mL - 78.01 µg/mL), verapamil (0.08 µg/mL - 45.5 µg/mL), FUMC (0.08 µg/mL - 40 µg/mL) and MK-571 (0.1 µg/mL - 50 µg/mL) were added. One row contained no cells and was used to normalize for background luminescence. A row containing cells to which no drugs were added was used as a control. The plates were incubated in a humidified incubator (37°C, 5% CO_2) for 72 hours.

Post-incubation, the plates were removed and allowed to equilibrate at RT for 30 minutes. A volume of 100 µl of CellTiter-Glo® reagent was added to each well. The contents were mixed on an orbital shaker for 2 min to induce lysis of the cells. The plate was then allowed to incubate at RT for 10 minutes to stabilize the luminescent signal. This luminescence was recorded by Tecan GENios microplate reader (Germany). The background luminescence was subtracted and the viability was calculated as a percentage of untreated cells assuming 100% viability.

6.2.6 Transcellular Permeation of Linezolid Across Caco-2 cells

Transwell plates were seeded with Caco-2 cells at 40,000 cells/well and incubated in a humidified incubator (37°C, 5% CO_2). Media was changed every other day for 21 days. On the day of the experiment, media was removed and replaced with HBSS containing 14 μg/mL linezolid alone or in combination with verapamil (P-gp inhibitor; 11.4 μg/ml; 25 μM), FUMC (BCRP inhibitor; 30 μg/mL; 79.06 μM) or MK-571 (MRP inhibitor; 20 μg/mL; 37.2 μM). HBSS containing the drugs were added to the apical (250 μl) or basolateral (550 μl) compartment and the receiver compartment contained HBSS. The plates were incubated in a humidified incubator (37°C, 5% CO_2). After 1 hour, aliquots of 150 μl were taken from the receiver compartment and sent for analysis in Cape Town as described below. Post-experiment, the integrity of the transwell was analysed by an assessment of the transepithelial electrical resistance (TEER). Additionally, [14C]-Mannitol was used to check the integrity of cells. Buffer from apical and basolateral chambers and washed 3 times with HBSS. A volume of 550 μl was added to the basolateral compartment, followed by 100 μl of [14C]-Mannitol solution to the apical compartment of the transwell (0.1 μCi/μL of mannitol in buffer). The plates were then incubated in a humidified incubator (37°C, 5% CO_2). After 1 hour, 100 μL of sample was taken from basolateral compartment and added to a vial containing 4mL of scintillation fluid. The radioactivity was measured by Packard Tri-Carb 3100 TA Liquid Scintillation Counter (Perkin-Elmer, Cambridge, UK).

6.2.7 Linezolid Cellular Accumulation in MDCK and MDCK-MDR1 cells

MDCK and MDCK-MDR1 cells were seeded in 4 wells of a 6-well plate at 1 x 10^6 cells/well for 24 hours in a humidified incubator (37°C, 5% CO_2). Media were then replaced with DMEM containing 10% FBS with linezolid at 6 μg/mL, 14 μg/mL or 21 μg/mL with or without verapamil (4.5 μg/mL; 10 μM; P-gp inhibitor) and incubated for 1 hour (37°C, 5% CO_2).[320] Post-incubation, the media was collected to determine extracellular linezolid concentration. Subsequently, cells were washed 3 times with cold HBSS to remove remaining culture media. Cells were removed from the culture plate by trypsinisation with 1mL of trypsin-EDTA per well and incubation for 10 minutes in a humidified incubator (37°C, 5% CO2). After incubation, the cells were agitated for detachment and transferred to vials to be sent for the measurement of intracellular substrate concentrations.

6.2.8 Digoxin Cellular Accumulation in MDCK and MDCK-MDR1 cells

Parallel accumulation experiments with digoxin were conducted in MDCK and MDCK-MDR1 cells to confirm the over-expression of P-gp in MDCK-MDR1 cells as compared to MDCK cells. The cells were seeded in 4 wells of a 6-well plate at 1 x 10^6 cells/well for 24 hours in a humidified incubator (37°C, 5% CO_2). Media were then replaced with DMEM containing 10% FBS with digoxin (7.8 μg/mL; 10 μM) with or without verapamil (4.5 μg/mL; 10 μM; P-gp inhibitor). Radioactive [3H]-digoxin was added along with digoxin at a concentration of 0.1 μCi/mL. The cells were then incubated for 1 hour (37°C, 5% CO_2). After incubation, 100 μl of the supernatant fraction was taken and transferred to a scintillation vial to determine the

extracellular substrate concentration. The cells were then washed 3 times with cold HBSS to remove the remaining culture media. Cells were removed from the culture plate by trypsinisation with 1mL of trypsin-EDTA per well and incubation for 10 minutes in a humidified incubator (37°C, 5% CO_2). After incubation, the cells were agitated for detachment and transferred to scintillation vials for measurement of intracellular substrate concentrations. A volume of 4 mL of scintillation cocktail was added to the vials and the radioactivity count was measured by a Packard Tri-Carb 3100 TA Liquid Scintillation Counter (Perkin-Elmer, Cambridge, UK).

6.2.9 Linezolid Bioanalysis

Linezolid concentrations were quantitatively determined using LC/MS/MS at the Division of Clinical Pharmacology, University of Cape Town.

Linezolid was extracted from a 100 µL sample using 200 µL of ACN containing a stable isotopic labelled internal standard (Linezolid-D3, 1 µg/mL). Samples contained Madin-Darby canine kidney epithelial cells (MDCK) or MDCK-MDR1 cells overexpressing P-gp transporters, both in DMEM containing 10% FBS. The extraction tubes were vortexed for 1 min, and centrifuged at 5590 g for 5 min. Two hundred microliters of the supernatant were removed and transferred to 96-well plates.

Chromatographic separation was performed on an Agilent 1200 High Performance Liquid Chromatography (HPLC) system comprising a binary pump, degasser, column compartment and an auto sampler (Agilent, Little Falls, Wilmington, USA). A reversed-phase HPLC column (Gemini NX C18 2.6 µm, 50 mm x 2.1 mm,

Phenomenex) was used with a pre-column filter (0.2 μm, Supelco). Mobile phases consisted of water containing 0.1% formic acid in line A and ACN containing 0.1% formic acid in line B. The mobile phases were delivered with a gradient of 5% ACN over 0.1 min, increased to 95% ACN over 1.9 min, held at 95% ACN for 1 min, dropped to 5% ACN in 0.6 min and held at 5% for 2.4 min for equilibration, with a flow rate of 0.5 mL/min for a total run time of 6 min. The column was kept at 20°C. Samples were cooled to 4°C whilst awaiting injection, and 5 μL was injected onto the analytical column.

Electrospray ionization was used in the positive ionization mode at unit resolution. The following multiple reaction monitoring transitions were monitored and the transition of the protonated precursor ions m/z 337.3 and m/z 340.5 to the product ions m/z 296.1 and m/z 297.3 were recorded for linezolid and linezolid-D3, respectively. The ion spray voltage was set at 4500 V. The source temperature was set at 300°C. The nebulizer gas, curtain gas and auxiliary gas pressures were set at 30, 55 and 45 arbitrary units, respectively.

Data acquisition and analysis were performed using Analyst 1.6.2 software. The calibration range was between 5 and 20,000 ng/mL. The accuracy (%Nom) and precision (%CV) statistics of the low, medium, and high quality controls (n=3) were between 97.8 and 112.3%, and below 15%, respectively.

6.2.10 Data Analysis

Cytotoxicity of drugs were calculated as percentage of cells alive after exposure to varying concentrations of drugs compared to cells in control group. The background luminescence was subtracted from the luminescence given out by cells exposed to drugs and in control group. Cell viability was then calculated using the formula:

$$\text{Cell Viability \%} = \frac{\text{Luminiscence of cells after exposure to drugs}}{\text{Luminiscence of cells in control}} \times 100$$

Apical to basolateral (A→B) and basolateral to apical (B→A) apparent permeability (P_{app}) was calculated using the formula:

$$P_{app} = \frac{(dQ/dt)}{(C_0 \text{ X } A)}$$

Where dQ/dt is the rate of drug permeation, C_0 is the drug concentration in the donor compartment at time 0, and A is the area of the monolayer.

Cellular accumulation ratio for linezolid and digoxin was calculated using the following formula (where DPM = disintegrations per minute):

$$CAR = \frac{(intracellular\ DPM/total\ cell\ volume)}{(extracellular\ DPM/extracellular\ volume)}$$

Cellular volumes were determined using the Scepter™ cell counter 2.0 (Merck Millipore, Billerica USA). Cell volumes were taken from a mean of 3 replicates, MDCK-MDR1 volume 3.7 pl.

Normality of data was assessed using a Shapiro-Wilk test. If data were not normal, log values were calculated and used to calculate statistical significance. An unpaired t-test was performed to assess statistical significance using SPSS 22.0. Statistical significance was defined as $P<0.05$. Figures were made using Graph-pad Prism (Version 6)

6.3 Results

6.3.1 Drug Toxicity

The drugs used in the study did not exhibit any cytotoxicity in MDCK (**Figure 6.1(A)**), MDCK-MDR1 (**Figure. 6.1(B)**) and Caco-2 (**Figure 6.1(C)**) cells. Cell viability was above 80% at all tested concentrations.

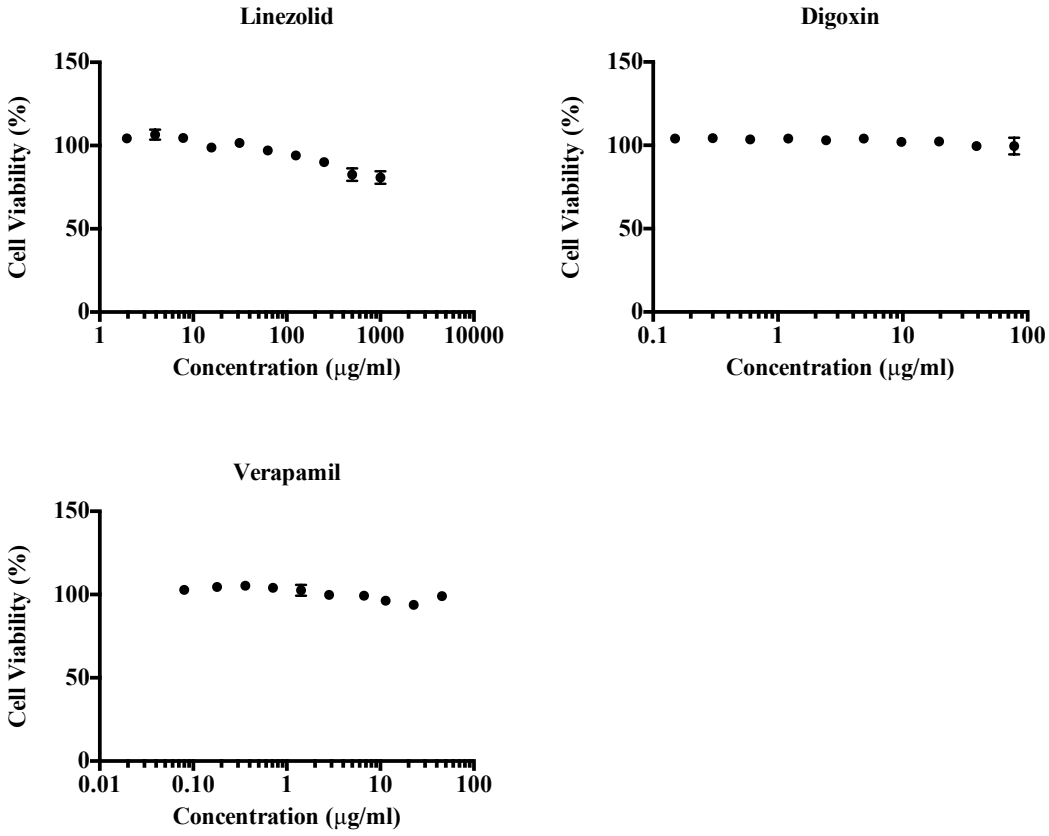

Figure 6.1 (A). Toxicity of linezolid (1000 μg/mL – 1.95 μg/mL), digoxin (78.01 μg/mL – 0.15 μg/mL) and verapamil (45.5 μg/mL – 0.08 μg/mL) on MDCK cells. n = 4.

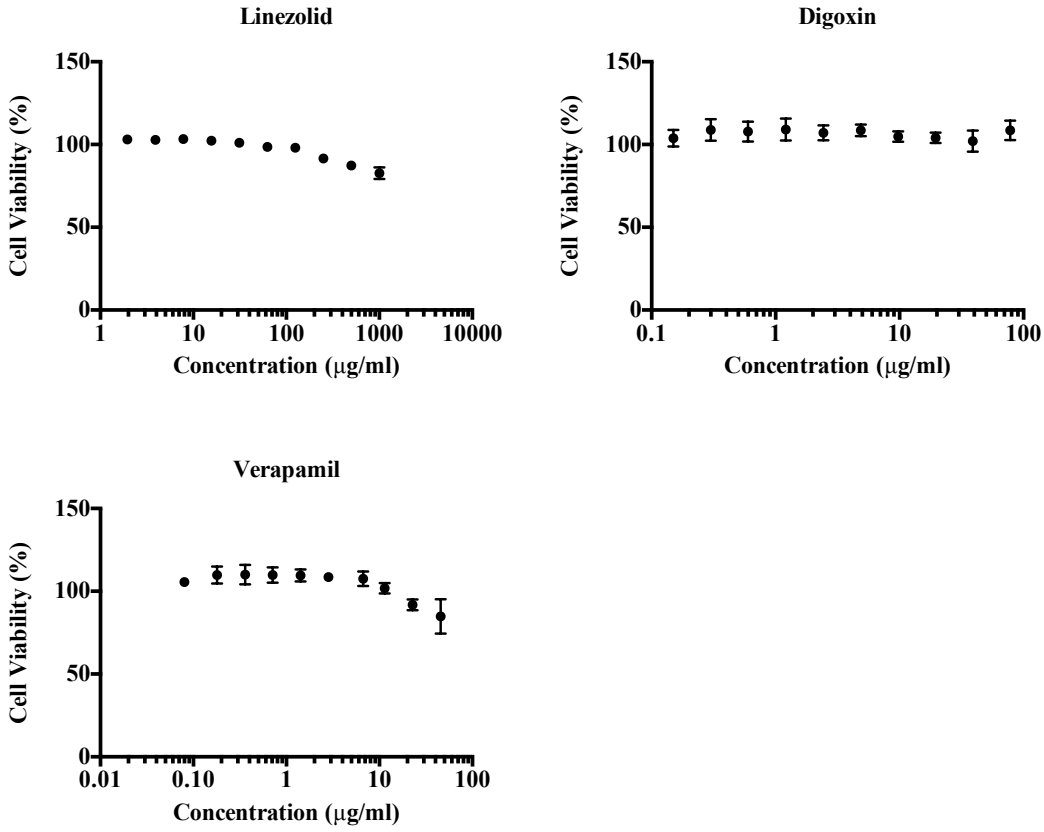

Figure 6.1 (B). Toxicity of linezolid (1000 μg/mL – 1.95 μg/mL), digoxin (78.01 μg/mL – 0.15 μg/mL) and verapamil (45.5 μg/mL – 0.08 μg/mL) on MDCK-MDR1 cells. n = 4.

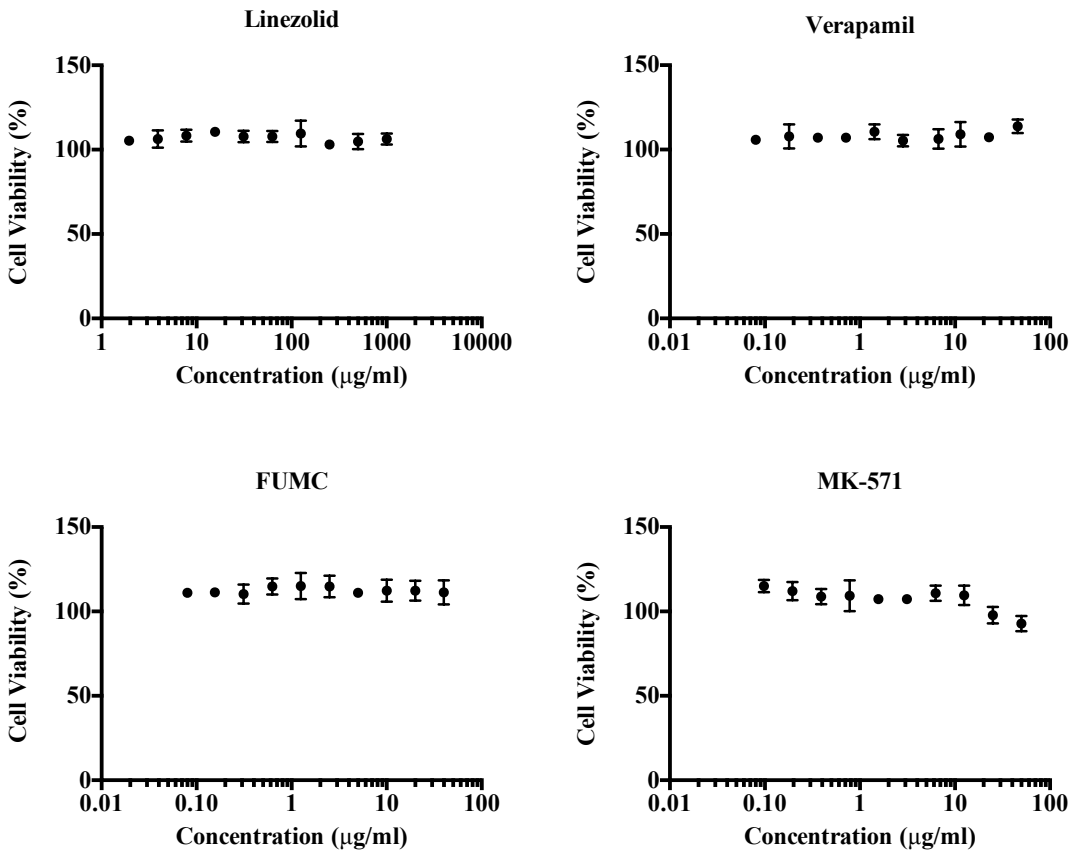

Figure 6.1 (C). Toxicity of linezolid (1000 μg/mL – 1.95 μg/mL), verapamil (45.5 μg/mL – 0.08 μg/mL), FUMC (50 μg/mL – 0.1 μg/mL) and MK-571 (40 μg/mL – 0.08 μg/mL) on Caco-2 cells. n = 4.

6.3.2 Transport of Linezolid Across Caco-2 Monolayers

At 1 hour, a significant decrease in A→B transport of linezolid (**Table 6.1**) was seen in the presence of verapamil (7.13×10^{-6}; $p = 0.007$) as compared to control (9.48×10^{-6}). There was a significant increase in A→B transport of linezolid when co-incubated with FUMC (12.62×10^{-6}; $p = 0.009$) and MK-571 (15.9×10^{-6}; $p = 0.0002$).

Verapamil did not have any statistically significant effect on the B→A movement of linezolid (19.16×10^{-6}; $p = 0.067$). The presence of FUMC (31.12×10^{-6}; $p = 0.001$) and MK-571 (37.51×10^{-6}; $p = <0.001$) led to an increase in B→A movement of linezolid compared to control (23.88×10^{-6}).

A significant reduction in the efflux ratio ($p = 0.022$) of linezolid was seen in the presence of MK-571. Verapamil and FUMC did not have any significant effect on the efflux ratio of linezolid.

Table 6.1. Change in the Papp A→B and Papp B→A transport of 14 μg/mL of linezolid at 1 hour in the presence of verapamil, FUMC and MK-571 across Caco-2 monolayers. Statistical significance by unpaired t-test. n = 4.

Inhibitor	Transporter	P_{app} A→B ($x10^{-6}$)	ST. Dev ($x10^{-6}$)	P Value	P_{app} B→A ($x10^{-6}$)	ST. Dev ($x10^{-6}$)	P Value	Efflux Ratio	P Value
Linezolid	**Control**	9.48	0.33	**N/A**	23.88	1.25	**N/A**	2.51	**N/A**
Linezolid + verapamil	**P-gp (ABCB1)**	7.13	0.86	**0.007**	19.16	3.48	0.067	2.68	0.336
Linezolid + FUMC	**BCRP (ABCG2)**	12.62	1.17	**0.009**	31.12	1.81	**0.001**	2.47	0.468
Linezolid + MK-571	**MRP (ABCC)**	15.90	0.88	**0.0002**	37.51	1.65	**<0.0001**	2.36	**0.022**

6.3.3 Cellular Accumulation of Linezolid with and without P-gp Inhibitor Verapamil

Verapamil did not influence the cellular accumulation of linezolid in MDCK cells (**Figure 6.2(A)**) at the tested concentrations of 6 μg/mL (p = 0.391), 14 μg/mL (p = 0.454) and 21 μg/mL (p = 0.323). Similarly, verapamil did not change the cellular accumulation of linezolid in MDCK-MDR1 cells (**Figure 6.2(B)**) at 6 μg/mL (p = 0.717) and 14 μg/mL (p = 0.114). However, a significantly higher cellular accumulation of linezolid at 21 μg/mL (p = 0.035) in MDCK-MDR1 cells was observed (**Figure 6.2(B)**).

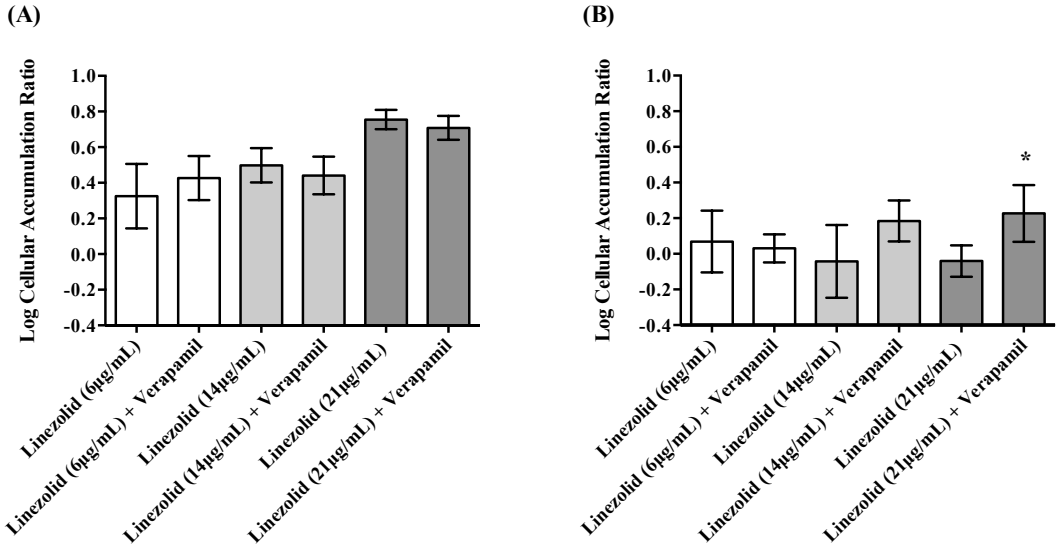

Figure 6.2. Cellular accumulation of linezolid at varying concentrations in MDCK (A) and MDCK-MDR1 cells (B) in the absence and presence of verapamil. Unpaired t-test used to establish significance for differences in linezolid accumulation. n = 4. P Value: *, < 0.05; **, < 0.01; ***, < 0.001; ****, ≤ 0.0001

6.3.4 Cellular accumulation of digoxin in the absence and presence of P-gp inhibitor verapamil

Cellular accumulation of digoxin (10 μM) in the presence and absence of verapamil (10 μM) were analysed (**Figure. 6.3**). It was seen that the cellular accumulation of digoxin was lower in MDCK-MDR1 cells compared to MDCK cells in the absence ($p = 0.0008$) and presence ($p \leq 0.0001$) of verapamil.

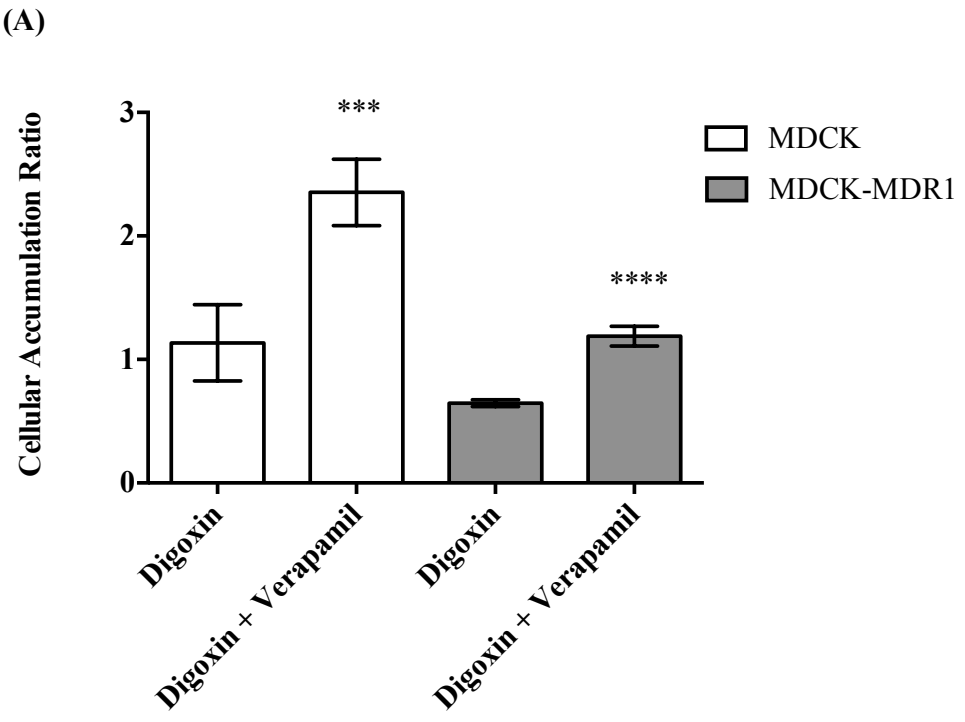

Figure 6.3. Cellular accumulation of digoxin (10 μM) in MDCK and MDCK-MDR1 cells in the absence and presence of known P-gp inhibitor verapamil (10 μM). Unpaired t-test used to establish statistical significance. P Value: *, < 0.05; **, < 0.01; ***, < 0.001; ****, ≤ 0.0001

6.4 Discussion

DDIs with linezolid have not been extensively studied, but case studies have described treatment failure on co-administration of rifampin[384] and selective serotonin reuptake inhibitors.[385] Bolhuis *et al*. have demonstrated an increase in linezolid exposure in the presence of clarithromycin, a potent P-gp inhibitor.[386] These findings warrant an investigation of the interaction of linezolid with transporters.

In the present study, verapamil decreased linezolid transport from A→B (**Table 6.1**), which is counterintuitive of an effect mediated by apically expressed P-gp, where an increase in A→B permeation would be expected. However, verapamil is known to inhibit a wide range of apical influx and basolateral efflux transporters such as such as OATP1A2[387] and OCT1[388] in Caco-2 cells.[389] This observation may indicate that linezolid is a substrate for additional transporters not investigated in this study. Due to these observations, the interaction of linezolid with P-gp was further investigated by assessing cellular accumulation in MDCK and MDCK-MDR1 cells. MDCK-MDR1 cells overexpress P-gp and this circumvents the problems of cross-interactions between multiple transporters in a non-transfected cell system.[313] A change in cellular accumulation of linezolid can be attributed to the change in activity of P-gp.

The over-expression of P-gp in MDCK-MDR1 cells was confirmed by cellular accumulation experiments using digoxin as a substrate. In MDCK-MDR1 cells, a significantly lower accumulation of digoxin with and without verapamil was seen compared to digoxin accumulation in MDCK cells, confirming the proper functioning of MDCK-MDR1 cells. At all concentrations tested, the accumulation of linezolid was lower in MDCK-MDR1 cells than in the parental MDCK cells (**Figure 6.2**). In MDCK-MDR1 cells, verapamil significantly inhibited linezolid efflux at 21 μg/mL

($p = 0.035$), but not at 6 μg/mL ($p = 0.717$) and 14 μg/mL ($p = 0.114$). This may again be explained by interference of other canine transporters within these cells that transport linezolid that may masking the influence of verapamil at lower concentrations. Nonetheless, the results demonstrate the influence of verapamil on linezolid, confirming that linezolid is a P-gp substrate.

The increase of linezolid transport from A→B in the presence of FUMC ($p = 0.009$) and MK-571 ($p = 0.0002$) can be attributed to the inhibition of BCRP and MRP efflux transporters, respectively, which are both situated on the apical surface of Caco-2 monolayers.[382]

Interestingly, the presence of FUMC ($p = 0.001$) and MK-571 ($p \leq 0.001$) also led to an increase in basolateral to apical (B→A) linezolid permeability. The integrity of the monolayers were assessed using an assessment of the TEER and [14C]-Mannitol and demonstrated to be within acceptable limits. It cannot be ruled out that these inhibitors also impact other transporters. FUMC has been shown to impact transporters other than BCRP,[390] and MK-571 is relatively non-specific to MRP isoforms[391] some of which are basolaterally expressed.[392] Nonethless, MK-571 significantly reduced the efflux ratio (2.36, $p = 0.022$) of linezolid compared to the control (2.51).

The International Transporter Consortium states that a molecule is potentially a P-gp or BCRP substrate if the if the efflux ratio is ≥ 2 in a cell system that expresses both the transporters. Moreover, if P-gp and BCRP inhibitors do not reduce the efflux ratio by 50%, other transporters maybe involved in the observed net flux.[314] Our findings show that the linezolid efflux was 2.51 indicating that it is a potential substrate of P-gp and BCRP. Moreover, the P-gp and BCRP inhibitors failed to reduce the efflux

ratio significantly indicating the involvement of other transporters affecting linezolid transport. These findings indicate that although linezolid is a substrate of P-gp and BCRP, involvement of other transporter may mean that these two transporters are less important *in vivo*.

A limitation of this study is that the tested transporter inhibitors are non-specific and can make it difficult to pin-point the exact transporter involved in the drug disposition. This was the first study done to assess the influence of P-gp, MRP and BCRP transporters on linezolid transport. The findings suggest the tested inhibitors influence the disposition of linezolid and provides a rationale for studies to assess linezolid transport by use of cells that are transfected and over-express a certain transporter, similar to the MDCK-MDR1 cells. Additionally, studies that determine the Vmax and Km of linezolid are required to determine the affinity of linezolid to a transporter.

In conclusion, this study showed that the BCRP inhibitor FUMC and MRP inhibitor MK-571 had a significant effect on the permeability of linezolid across Caco-2 cell monolayers. The demonstration that linezolid is a P-gp substrate may explain the low serum concentrations of this drug when co-administered with rifampicin; a P-gp inducer that facilitates the clearance of P-gp substrates.[384] Further studies are would be required to pin-point the mechanisms involved in the transport of linezolid by other transporters.

Chapter 7

General Discussion

Although there is currently no cure for HIV, remarkable strides in the past couple of decades have reduced the spread of the disease. Strategies such as raising awareness about HIV, prompting HIV testing and counselling of patients have played an important role in curbing the spread of infection.[393] However, the benefits of pharmacological interventions on limiting new HIV infections as well as improving the morbidity and mortality of individuals infected with HIV are unparalleled.[394] HAART reduces the viral load in infected individuals, preventing damage to $CD4^+$ cells and loss of immunity.[395] Early initiation of HAART is also associated with decreased transmission of HIV on exposure,[396,397] and a significant reduction in AIDS-defining and non-AIDS-defining illnesses.[398] As a result of these findings, the WHO 2015 guidelines recommend initiation of multidrug ART for all HIV infected individuals regardless of the WHO clinical stage or $CD4^+$ T-cell count.[258] Additionally, use of HAART for PrEP for the reduction of HIV transmission in high-risk populations is promising.[399]

Notwithstanding the advantages, treatment with HAART is not without challenges. Individuals infected with HAART must adhere to a strict HAART regimen throughout their lives for effective treatment, and missed doses can give rise to resistance to the ARVs thereby limiting the options for subsequent treatment.[92] Long term ARV administration is costly,[400] requiring permanent changes in lifestyle[401] and can lead to debilitating conditions due to drug toxicities.[86]

The pharmacokinetics and pharmacodynamics of drugs depend on a host of factors (**Figure 7.1**). ART is often combined with other drugs used to treat OIs and concomitant illnesses. Presence of an inducer/inhibitor of metabolic enzymes and transporters, or plasma protein displacers, can commonly result in DDIs.[259] The excipients used in formulations have also shown to exert biological effects on drugs

and are being exploited to achieve favourable drug PK. Furthermore, genetic variability in patients affecting the drug metabolism and disposition can affect drug PK, affecting treatment outcomes.[165] Understanding these influence on drug PK are paramount for designing rational therapies.

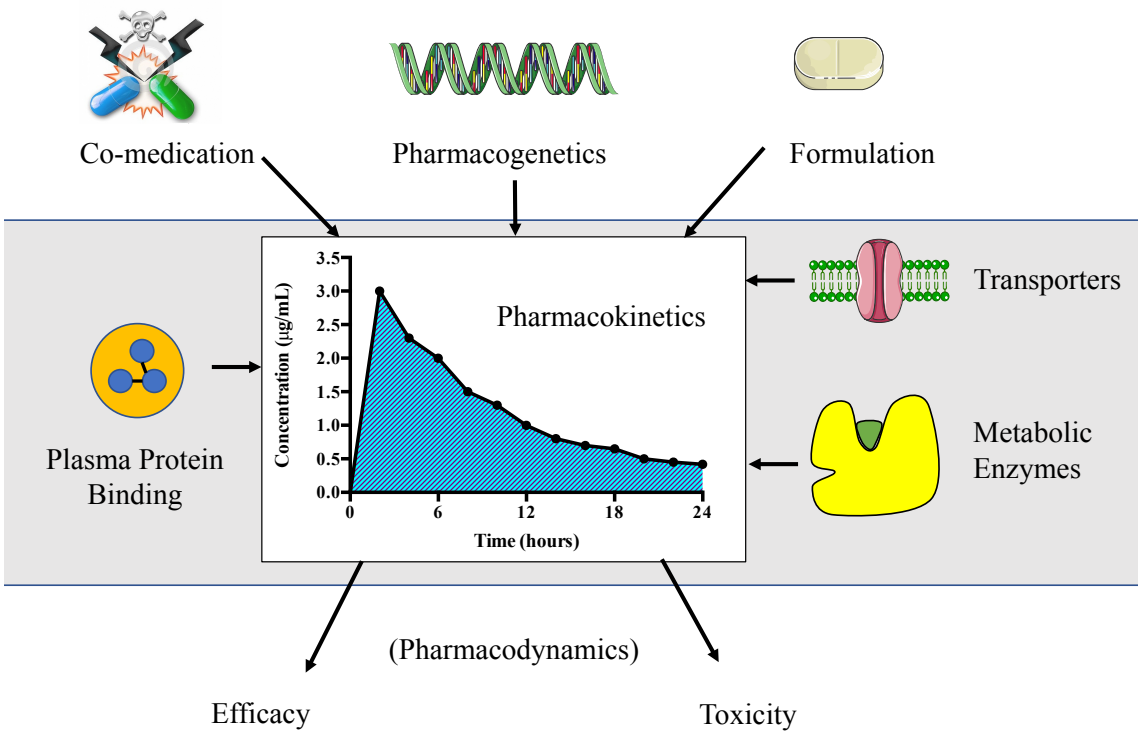

Figure. 7.1 Graphical representation of the factors contributing to the pharmacokinetics and pharmacodynamics of a drug

The use of 2 NRTIs as a backbone along with a third agent from a different class has been recommended in many guidelines for the treatment of HIV.[80,102] However, some of the most important concerns faced when administering NRTIs are toxicity and cross resistance. For example, zidovudine is associated with anaemia, hepatotoxicity and cardiomyopathy and is associated with cross resistance.[402] Similarly, tenofovir is associated with mitochondrial toxicity affecting glomerular filtration, creatinine

secretion and bone density.[226] With the development of superior ARVs, such as second-class PIs and integrase inhibitors, regimens without NRTIs are currently being tested for the treatment of HIV. A Phase III, randomized, open labelled trial called NEAT 001 / ANRS 143, was conducted to analyse the efficacy of a novel NRTI-sparing dual combination of darunavir/r and raltegravir against a standard-of-care triple therapy of darunavir/r and TDF/emtricitabine in HIV-infected antiretroviral naïve subjects. One objective of this study was to investigate the effect of SNPs on the drugs administered in this clinical trial; described in Chapters 2 and 3. This study contains the largest sample size of participants to date, in which specific SNPs have been tested and shown to have a clinically-significant association with darunavir, ritonavir, tenofovir, emtricitabine and raltegravir plasma concentrations.

The pharmacokinetic profile of darunavir and raltegravir are exclusive to each other, suggesting a lack of DDIs between the two when co-administered. However, darunavir concentrations were lower in patients receiving darunavir/r with raltegravir compared to darunavir/r with tenofovir and emtricitabine. An interaction between darunavir and tenofovir has been suggested by Hoetelmans *et al*. who demonstrated an increase in the C_{max}, AUC and C_{min} of darunavir by 16%, 21% and 24% respectively, in the presence of TDF.[200] The mechanism of this interaction is unknown. Conversely, an interaction between darunavir and raltegravir was reported in multiple studies in the past.[201,202] Similarly, ritonavir concentrations were seen to be significantly lower in patients receiving darunavir/r with raltegravir compared to darunavir/r with tenofovir and emtricitabine. Since ritonavir and darunavir are PIs and share similar pharmacokinetic properties, the mechanisms causing DDIs with darunavir may also be responsible for DDIs with ritonavir. The exact mechanism of this DDIs cannot be hypothesised from the current data on the pharmacokinetics of

protease inhibitors and raltegravir. Additional studies are needed to characterise the profile of these drugs.

The results from the NEAT001/ANRS143 study showed a higher percentage of treatment failure in the patients receiving raltegravir compared to those receiving TDF/emtricitabine and is correlated to below detectable levels of darunavir.[110] Moreover, a significantly higher number of mutations were seen in patients receiving raltegravir compared to no mutations in patients receiving TDF/emtricitabine.[204]

With the exception of *SLCO1B1* 521T>C (rs4149056) and *SLCO3A1* G>A (rs4294800), none of the tested polymorphisms were found to significantly affect the drug plasma concentrations. *SLCO1B1* 521T>C resulted in significantly higher ($p = 0.038$, $\beta = 0.075$) concentration of darunavir at week 4. Similarly, *SLCO3A1* G > A (rs4294800) was associated with higher ritonavir plasma concentrations at week 4. This polymorphism was chosen from a Physiologically based pharmacokinetic (PBPK) model that investigated darunavir PK in pregnant women has suggested involvement of hepatic transporters in the disposition of darunavir. Molto *et al*. using a pop-PK-model, showed a significant effect of *SLCO3A1* G > A (rs4294800) and *SLCO3A1* G > T (rs8027174) polymorphisms, that code for OATP3A1, on darunavir clearance and apparent volume of distribution.[190] To our knowledge, the transport of darunavir via OATP3A1 has not been demonstrated by *in vitro* experimentation. Robust experiments that confirm the substrate recognition of darunavir by the OATP3A1 transporter.

Multivariate regression analysis did not show associations between *UGT1A1**28 and raltegravir plasma concentration. However, a P value of 0.5 with β of 0.115 indicates a trend towards higher raltegravir plasma concentrations in the presence of

*UGT1A1**28 allele. Raltegravir has high intra-patient variability and a single time point collection was taken at week 4 and week 24 will not be precise to evaluate the influence of genetic polymorphisms. A co-relation between the pharmacodynamic properties of raltegravir such as treatment efficacy and adverse events will be more accurate to describe the influence of *UGT1A1*28*.

Although these findings are observed in clinical samples, they do not reflect the effect of SNPs on treatment. The results of the NEAT 001/ ANRS 143 show that darunavir/r + raltegravir was non-inferior to darunavir/r + tenofovir + emtricitabine.[110] An analysis of the influence of SNPs on not only the PK, but also the adverse events are warranted for an accurate representation of the effects of genetic polymorphisms. A precaution to be taken while interpreting these results is that due to strict inclusion and exclusion criteria, the participants in the study may not be fully representative of patients in a clinical setting. Management of HIV is complicated and involves variables such as co-morbidities, co-medications and altered physiologies. Moreover, NEAT 001/ANRS 143 being a multi-centre study, it is possible that factors that could cause a significant impact on drug PK and is restricted to an environment have not been included in the analysis. For example, the herbal preparations included in the Indian medicine 'Ayurveda' may result in significant DDIs with ARVs, influencing ART. Hence, these findings though informative, may not be universally applicable.

Investigations into the genetic variability provide an important tool to personalise medicine as it helps predict the efficacy of a treatment and the adverse effects in patients.[403] Recently, data on genetic variability is being incorporated into designing clinical trials to assess the efficacy of drugs on translation from bench-to-bedside. Adding genetic genotypes into the selection criteria helps assessing the influence on different groups based on their genotype.[404] For example, the trial investigating the

effects of a 5-lipooxygenase-activating protein inhibitor on biomarkers of myocardial infarction enrolled patients containing the at-risk variants in the FLAP gene.[405]

There are thousands of polymorphisms that are found in genes that code for proteins responsible for drug metabolism and disposition and most of them do not have any significant changes on the drug PK. However, some polymorphisms have a significant impact warranting dose adjustment. For example, the presence of *CYP2B6*6* allele, commonly found in black populations, is responsible for increased plasma concentration of efavirenz and adverse events. Vigilance during administration of efavirenz to black populations and dose-adjustments for people carrying this allele is recommended.[406]

The effects of induction and inhibition of metabolic enzymes and transporters on DDIs have been well-established. However, there is a lack of consensus on the role of plasma protein binding and displacement on DDIs.[154] Displacement of a drug from the plasma protein will increase the unbound fraction and could result in an increase in C_{max}, potentially causing toxicity and therefore requiring dose adjustment.[272] Additionally, higher unbound concentrations increase the clearance of the drug resulting in a drop in C_{min} below MEC.[273] These changes may result in treatment failure. Although the displacement from plasma proteins have been demonstrated *in vitro*, their clinical significance is routinely questioned.[283] In chapter 4, the level of plasma protein binding of darunavir, atazanavir, ritonavir and lopinavir was measured along with their displacement using known AAG and albumin displacers. This data will improve clarity in the evaluation of the clinical significance of plasma protein binding measurements.

The findings showed that darunavir (14.2%) and atazanavir (8.4%) unbound percentages differ from the values stated in previous literature (3.5% and 14% for darunavir and atazanavir respectively).[275,282] These corrected values will help in accurate representations of the pharmacokinetic profiles such as clearance and volume of distribution, for darunavir and atazanavir. It will also help in precise predictions of drug effects in future PBPK modelling efforts. Lopinavir and ritonavir are unbound at 1.1% and 0.7% respectively, which is similar to the values reported in previous literature. We also found that all the PIs bind to both AAG and albumin at the bilirubin site.

When both AAG and albumin displacers were combined, a greater increase in the unbound percentage was seen. This may represent an evolutionary mechanism to prevent high unbound concentrations of substances that could result in toxicity, and may explain why protein binding displacement rarely causes significant clinical DDIs.[154] However, it is possible that in cases of co-morbidities or altered physiologies that cause changes in AAG and albumin concentrations, displacement from plasma proteins could result in clinical significant DDIs. Vigilance over displacement DDIs in individual cases may therefore be required.

Our study used HIV non-infected plasma compared to the HIV-infected individuals in previous studies which might explain the discrepancies in the results. A thorough investigation into the changes of plasma protein binding due to HIV infection and its impact on protein binding is warranted. Furthermore, the reproducibility of these results with different displacers need to be assessed.

This data assist pharmacologists in designing drugs that are less susceptible to DDIs. For example, drugs with low protein binding and bind to both AAG and albumin,

when displaced, will have a low impact on the unbound fraction and drug pharmacology. A well-characterised profile of the plasma protein binding of drugs will also help PBPK modelling as it is an important variable to predict volume of distribution and clearance.[407]

The inertness of excipients was disproved by studies that demonstrated changes in the transporter-mediated absorption of substrates[292,294] and alterations in CYP enzyme activity.[295,296] This adds new modalities for potential DDIs. P-gp is an efflux transporter[303] and a change in its activity through inhibition or induction by excipients can cause significant changes in the disposition and PK of substrate drugs. A thorough investigation of the effects of excipients on P-gp is beneficial to predict changes in the PK of drugs. Importantly, these properties can be exploited to design rational formulations by which the excipients can be used as pharmacoenhancers. For example, Vit-E-PEG is increasingly used in formulations to increase the bioavailability of drugs by inhibiting P-gp.[325,326] In Chapter 5, we analysed the concentration-dependent inhibitory effects of 25 commonly used excipients on P-gp by measuring the accumulation of P-gp probe substrate digoxin in MDCK-MDR1 cells in the presence of the excipients.

The inhibition of P-gp by commonly-used pharmaceutical excipients was demonstrated by the following order of effects: Cremophor EL > Vit-E-PEG > Brij 58 > Tween 80 > NaCMC > Tween 20 > CTAB > Solutol HS 15 > AOT. The P-gp inhibition by Vit-E-PEG,[326] Cremophor EL,[408] AOT,[342] Tween 20, [334] Tween 80,[336] CTAB[344] and Solutol HS 15[332] have been described in the past and were confirmed in this thesis. Moreover, the presented data represent the first-time P-gp inhibitory effects of Brij 58 and NaCMC have been demonstrated *in vitro*. No inhibition of P-gp was observed with NaCap, NaDC, PEG, PVA, HPMC, HG, PVP K30, Sisterna

11, Sisterna 16, Hyamine, HPC, Kollicoat, PVPP and magnesium stearate. To our knowledge, there are no published studies to date that state otherwise. It must be noted that cytotoxicity was observed with NaDC, AOT, Brij 58, Hyamine, CTAB, NaCMC and Sisterna 16.

The biological effects of the excipients can be exploited to boost the pharmacokinetic properties of drugs. Such characterisations will aid the manufacturers in the choice of excipients in while designing formulations. The effect so these excipients on other transporters needs to be explored.

The decrease in the CD4+ cell count weakens the immune system and renders the body helpless against OIs such as TB and therefore needing treatment. ART and ATT consists of multiple drugs that cross-interact resulting in DDIs. These drugs are highly toxic and the DDIs observed have been co-related to low adherence, treatment discontinuation and MDR-TB.[22] It is important to have a well-defined profile of drugs present in both ART and ATT. Linezolid, an oxazolidinone, is found to be active against *Enterococcus faecalis, Staphylococcus aureus, Chlamydia pneumoniae, Haemophilus influenza* and TB.[174,370] It has good bioavailability, no cross resistance[371] and a high genetic barrier to resistance[372] making it popular against MDR-TB. To ensure efficient use, it is important to explore all the pharmacological properties of linezolid. Linezolid is suspected to be a P-gp substrate, but no data currently exist to show substrate affinity for P-gp or other ABC transporters that mediate DDIs and resistance.[380] More drug transporter data are therefore needed to inform DDI prediction and management. Accordingly, in Chapter 6, the substrate recognition by P-gp, BCRP and MRP transporters was investigated. The transporter substrate properties of linezolid was assessed across Caco-2 monolayers in the presence or absence of P-gp, MRP and BCRP inhibitors verapamil[381], MK-571[382] and

FUMC,[383] respectively. To further corroborate these results, linezolid accumulation was measured in MDCK-MDR1 cells.

Verapamil, MK-571 and FUMC all influenced either A→B or B→A transport of linezolid across Caco-2 membranes. However, this effect was not in line with the expected effects of inhibiting P-gp, MCRP and MRP. To futher explore this, linezolid accumulaton in the presence of verapmail was tested in MDCK-MDR1 cells. A significant increase in intracellular concentrations confirmed that linezolid was a substrate of P-gp. We hypothesise that this variation is due to the infidelity in transpoter inhibition shown by verapamil, MK-571 and FUMC. Verapamil is known to inhibit a wide range of apical influx and basolateral efflux transporters such as such as OATP1A2[387] and OCT1[388] in Caco-2 cells.[389] Similarly, FUMC has been shown to impact transporters other than BCRP,[390] and MK-571 is relatively non-specific to MRP isoforms[391] some of which are basolaterally expressed.[392]

These findings show that linezolid transport is affected by verapamil, MK-571 and FUMC, however, the exact transporter involved remains unclear. Additional studies are required to confirm transport in the presence of an inhibitor that is specific to a transporter, or cells lines that over-express the specific transporters. This will help design superior anti-tubercular therapies and help predict DDIs when co-administered with other drugs.

In conclusion, *SLCO1B1* 521T>C (rs4149056) and *SLCO3A1* G>A (rs4294800) is associated with a significant increase in the darunavir and ritonavir plasma concentrations respectively. Darunavir and ritonavir seem to interact with either tenofovir or raltegravir and the pharmacokinetic properties of these drugs need further exploration. PIs bind to both AAG and albumin and when co-administered with

concomitant drugs could give rise to DDIs in special populations. Cremophor EL, Vit-E-PEG, Brij 58, Tween 80, NaCMC, Tween 20, CTAB, Solutol HS 15 and AOT inhibit P-gp and this needs to be considered while choosing excipients. Verapamil, MK-571 and FUMC influenced linezolid permeability and further studies are required to narrow down the transporters responsible for it. These findings will assist development of treatment strategies to ensure optimal treatment outcomes.

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